# Behavioural analysis of 6-hydroxydopamine rodent models of Parkinson's disease

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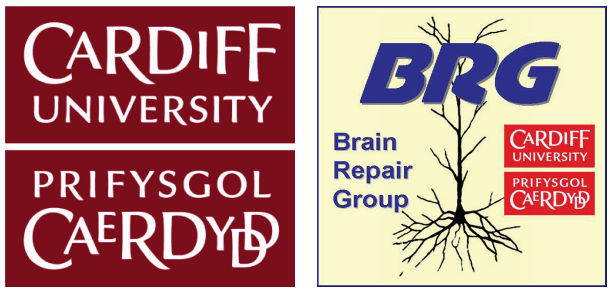
*This dissertation is submitted for the degree of*

*Doctor of Philosophy at the*

*University of Cardiff*




**Cardiff University**

**November, 2012**

*I dedicate this thesis to my parents, Johanna and Klaus Heuer.*

*Thanks mom and dad for all your love and support!*

# Summary

The aims of this thesis were to characterise further lesion-induced impairments in unilateral rodent models of Parkinson's disease (PD) on a more cognitive level and to investigate the effects of cell replacement therapies on these tests.

**Chapter 3.1** deals with the effects of dopamine depletion on a lateralised choice reaction time task in the Skinner box as this apparatus is more widely available than the 9-hole boxes on which initial studies have been based. Unilateral near complete lesions of the nigro-striatal pathway induced a stable side bias that was comparable to the lesion-induced deficits that have been reported in the 9-hole box apparatus. **Chapter 3.2** reports on the effects of similar lesions on a more spatial reaction time task and the effects of engraftment of dopamine rich tissue in the denervated striatum. The lesions induced a spatial bias that was only marginally improved by the cell transplantation, clearly showing the limitations of ectopic graft placement. Nevertheless, small but significant improvements on that task could be shown as grafted animals performed with higher accuracy and had reduced movement times compared to the lesion only counterparts. **Chapter 3.3** explores the lesion-induced deficit in more detail by implementing an error correction rule on the operant task to enforce a change in the animals' response strategy. The results of this chapter confirmed earlier findings, that the dopamine depletion produced by the lesion gives rise to a strong near hole bias on the contralateral side which did not recover, even with extensive post lesion testing, i.e. the lesion-induced deficit is most likely to be caused by a misrepresentation of response space, rather than caused by a shift in response strategy.

The second strand of this thesis focuses on the development of mouse models of similar dopamine-depleting lesions that are typically used in rat models of PD. In **Chapter 3.4** the three most common lesion models are compared to each other on an extensive battery of simple motor tests. The aim was both to characterise the behavioural impact of dopamine depletion in different sites, as well as to identify appropriate hand tests, capable of distinguishing lesions greater than 70% depletion. The differences and similarities between lesions were evaluated and correlations between behavioural performance and nigral cell loss were observed. In **Chapter 3.5** I developed parameters which allowed application of the lateral choice reaction time task to mouse models of dopamine depletion. Here I demonstrate the effects of two lesions, either to the medial forebrain bundle or the substantia nigra, on the same task conducted in mice. Lesioned mice of the former group displayed a stronger deficit largely because of the larger dopamine depletion. Subsequently, in **Chapter 3.6** I characterise the effects of primary fetal tissue grafts on the previously established model and task. Primary fetal tissue was able to ameliorate some of the lesion-induced deficits on an operant choice reaction time task and a series of simple motor screens.

The results of both strands of research in the present thesis have implications for the understanding of the cognitive and motor deficits that are induced by the most commonly used lesion model of PD and for the parameters that can be recovered by cell replacement therapies. The primary fetal tissue will serve as a baseline, against which future stem cell based therapies can be measured.

# Declaration

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

Signed: …… Date: 27/12/2012

**STATEMENT 1**

This thesis is being submitted in partial fulfillment of the requirements for the degree of PhD.

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**STATEMENT 2**

This thesis is the result of my own independent work/investigation, except where otherwise stated. Other sources are acknowledged by explicit references. The views expressed are my own.

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**STATEMENT 3**

I hereby give consent for my thesis, if accepted, to be available for photocopying and for inter-library loan, and for the title and summary to be made available to outside organisations.

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# Foreword on the format of this thesis

Following planning the submission of this thesis my supervisor Professor Stephen B. Dunnett suggested to submit the present work as a series of self-contained publications which have either been accepted for publication, are submitted and currently under review, or are in preparation for submission. This format of thesis submission has been adopted in agreement with the former head of graduate studies Professor Vic Duance, Head of Biosciences and the University of Cardiff.

The submission by paper format has the advantages of (i) conducting and planning experiments that are suitable for submission to journals, (ii) all of the results of the work conducted can be cited and used for future work already at the time of thesis submission, (iii) publications prior to submission are highly advantageous for the candidate's CV as they can be a proof of productivity, (iv) the process of peer-review and manuscript submission, an important component in the overall scientific process of bringing experimental results into the public domain and are, in my opinion, essential for the education of a young researcher, (v) peer-reviewed publications are essential in many European countries to obtain a PhD and therefore submitting in the paper format is important when applying for international jobs in countries that are not familiar with the U.K. system, and (vi) the critical process offered by peer-review has the potential to strengthen my writing of the submitted work and to aid my preparation for the thesis defence, as the ideas and results contained therein will already have been scrutinised - and where necessary challenged - by experts in the field.

The general introduction and discussion as well as the methods sections are presented in the conventional thesis format, but they are reduced in length as the individual manuscripts will provide full information about the respective procedures. Some of the presented publications have multiple authors as conducting scientific research depends on collaboration, interaction and the exchange of ideas. In the introduction of each experimental chapter a brief statement provides the information about the input of each author in the presented experiment. Especially in experimental chapter 3.4 I fully acknowledge the close collaboration with Dr. Gaynor Ann Smith, a former fellow PhD student within the same research group in Cardiff.

# Acknowledgements

My deepest gratitude goes to Steve Dunnett and Anne Rosser who have kindly allowed me to play in their laboratory for four years. Steve, you have been a great supervisor, mentor and friend. I cannot thank you enough for the opportunity and guidance you have given me!!! Thank you!!!

Many thanks as well to Mariah Lelos who has supervised me along this PhD experience and whose doors were always open, thank you for going the extra mile more than once!

Arjan Blokland, who sent me to Cardiff in the first place. Arjan you were a great mentor during my time in Maastricht and inspired me to become fascinated with the brain in the first place. Thank you very much!

Simon, what can I say… thanks for all the mentoring and support on and off the pitch! Thanks for all the talks, discussions, advice, and football!

Many, many thanks to the people who supported me especially during the final weeks of writing for reading and correcting: Amy, Sophie, and Alex!!! Diolch!!! As well to my current office mates for putting up with me and keeping the noise down during the last couple of weeks!

I had an amazing time here at the BRG which is only due to the amazing people that work here, my former and current office mates and fellow PhD students who have become friends: Nari, Carl, Gaynor, Sophie, Zubeyde, Dr. Steve, Nan, Amy, Harri, Vicky, Ulrike, Susannah, Lu and Claris. Thank you all for your friendship, help and support, as well as the many cups of tea and cake! Nobody could have asked for a better group of people to share this experience with. All of you will truly be missed! The post-docs, Mate, Claire, Emma, Bex, Hanna, Alex, Rike, Ed and Simon: really glad that I have met all of you. You are truly inspirational people and I and very thankful that I could always come to you for advice. Alex, boy how do I miss our German nights!!!

To the people who make the lab run the way it is: Anne-Marie, Ali, Jane and Ngoc-Nga, Anna and Gemma. Thank you for all the hours of support.

Ma & Pa, Opa, Katrin, Thank you guys for all your support from my first move to Holland, during my time in Australia, to the experience in Wales. I could never have done anything without knowing of your support. I have missed you all the way and looking forward seeing you more often.

# Index

# Summary of papers presented in this thesis

This thesis contains six original manuscripts on the subject of characterisation of the unilateral 6-OHDA lesion model of Parkinson's disease for the assessment of cell replacement therapy.

**Heuer A, Dunnett SB:**
Unilateral 6-OHDA lesions induce lateralised deficits in a 'Skinner box' operant choice reaction time task in rats.
*Journal of Parkinson's Disease.* 2(4), (2012): 309-320.

**Heuer A, Lelos MJ, Kelly CM, Torres EM, Dunnett SB:**
Graft-induced amelioration of lateralised response bias induced by unilateral nigro-striatal 6-OHDA lesions in rats.
*Experimental Neurology*. (2013). *In press.*

**Heuer A, Dunnett SB:**
Characterisation of spatial neglect induced by unilateral 6-OHDA lesions on a choice reaction time task in rats.
*Behav. Brain Res.* 237, (2013): 215-222.

**Heuer A*, Smith GA*, Lelos MJ, Lane EL, Dunnett SB:**
Comprehensive Behavioural Evaluation of Striatal, MFB and Nigral Unilateral 6-ohda Lesioned Mice (Part I).
*Behav. Brain Res*., 226(1), (2012): 281-292.

**Heuer A, Smith GA, Dunnett SB:**
Comparison of 6-hydroxydopamine lesions of the substantia nigra and the medial forebrain bundle on a lateralised choice reaction time task in mice.
*Eur J Neurosci*. , 37, (2013), 294-302.

**Heuer A, Vinh NN, Dunnett SB:**
Behavioural recovery on simple and complex tasks by means of cell replacement therapy in mice.
*Eur J Neurosci.,* 37, (2013): 1691-1704.

* Both authors contributed equally

## Summary of additional publications

The nature of working in science allows for the collaboration and exchange of ideas. During my doctoral studies I had the privilege to work with many excellent scientists, both friends and collagues, within the Brain Repair Group as well as with several of Professor Dunnett's international collaborators. I was lucky enough that some of those collaborations were fruitful and led to publications that are related to the present work, which are outlined below.

**Smith GA*, Heuer A*, Dunnett SB, Lane EL:**
Unilateral 6-OHDA lesions of the Striatum, MFB and Nigra in Mice (Part II). Analysis of the Behavioural and Histological Hallmarks of L-dopa Induced Dyskinesia. *Behav. Brain Res.,* 226 (2012) 28-292.

**Smith GA, Heuer A:**
The 6-OHDA Lesioned Mouse Model of Parkinson's Disease. In Lane EL and Dunnett SB (eds), *Animal Models of Movement Disorders, Volume I.* Springer Protocols, Humana Press, New York (2011). pp. 281-287.

**Smith GA, Heuer A, Klein A, Vinh NN, Dunnett SB, Lane EL:**
Amphetamine-induced dyskinesia in the transplanted hemi-parkinsonian mouse. *Journal of Parkinson's Disease,* 2 (2012) 107-113.

**Torres EM, Lane EL, Heuer A, Smith GA, Murphy E, Dunnett SB:**
Improvement of the 6-Hydroxydopamine lesion of the median forebrain bundle by modification of the stereotaxic coordinates. *J. Neurosci. Meth.,* 200(1), (2011) 29-35.

**Brooks SP, Janghra N, Higgs GV, Bayram-Weston Z, Heuer A, Jones L, Dunnett SB;** Selective cognitive impairment in the YAC128 Huntington's disease mouse. *Brain Research Bulletin,* 88(2-3), (2012): 121-129.

**Dunnett SB, Heuer A, Lelos M, Brooks SP, Rosser AE:**
Bilateral striatal lesions disrupt performance in an operant delayed reinforcement task in rats. *Brain Research Bulletin,* 88(2-3)), (2011): 251-260.

**Arber C, Precious SV, Kelly CM, Heuer A, Chang, KH, Rodriguez TA, Rosser AE, Dunnett SV, Li M;**
A Novel strategy for generating transplantable striatal GABAergic projection neurons from human pluripotent stem cells. *Submitted to Nature Biotech.* (2012)

**Brooks SP, Heuer A, Dunnett SB;**
Assessment of motivation and impulsivity in mouse models of Huntington's disease. *In preparation*

**Precious SV, Heuer A, Smith GA, Kelly CM, Jaeger I, Rosser AE, Li M, Dunnett SB**; Behavioural analysis of dopaminergic cells derived form the mouse epiblast under defined factors.
*In preparation*

**Cerovic M. Bagetta V, Prndolino V, Ghiglieri V, Fasano S, Morella I, Hardingham N, Heuer A, Papale A, Marchisella F, Giampa C, Calabresi P, Picconi B, Brambilla R**. Ras-GRF1 and ERK mediated long-term potentiation at the cortico-striatal synapse is altered in L-DOPA induced Dyskinesia. *Submitted to Brain. (2013).*

# Abbreviations

The abbreviations listed below will be used throughout this thesis. Abbreviations other than these will be clearly labelled in the text/figure legend.

| | |
|---|---|
| 5-HT | Serotonin |
| 6-OHDA | 6-hydroxydopamine |
| ACh | Acetylcholine |
| AChE | Acetylcholinestherase |
| ANOVA | Analysis of Variance |
| AP | Anterior-Posterior |
| BDNF | Brain Derived Neurotrophic Factor |
| BBB | Blood brain barrier |
| CV | Cresyl Violet |
| DA | Dopamine |
| DARPP-32 | Dopamine and cyclic AMP-regulated phosphoprotein |
| DAT | Dopamine transporter |
| DMEM | Dulbecco's modified eagle's medium |
| DV | Dorsal-Ventral |
| E | Embryonic Day |
| EpiSC | Epiblast derived stem cell |
| ESC | Embryonic stem cells |
| GABA | γ-Aminobutyric acid |
| GFP | Green flourecent protein |
| GPi/e | Globus pallidus (internal/external) |
| HD | Huntington's disease |
| iPSC | Induced pluripotent stem cells |
| ITI | Inter trial interval |
| LX | Post lesion |
| ML | Medial-Lateral |
| MFB | medial forebrain bundle |
| MSNs | Medium spiny neurons |
| NeuN | Neuronal nuceli |
| PBS | Phosphate buffered saline |
| PD | Parkinson's disease |
| PFA | Paraformaldehyde |
| SN | Substantia nigra |
| SNpc | Substantia nigra pars compacta |
| STN | Subthalamic nucleus |
| TH | Tyrosine-hydroxylase |
| TBS | Tris-buffered saline |
| TNS | Tris Non Saline |
| TOI | Time out interval |
| TX | Post graft |
| TxTBS | Triton X-100 TBS |
| UPDRS | Unified Parkinson's disease rating scale |
| VMAT | Vesicular monoamine transporter |
| VTA | Ventral tegmental area |
| wGE | whole ganglionic eminence |

# Chapter 1 General Introduction

## 1.1 What is Parkinson's disease?

Parkinson's disease (PD) was firstly described by the surgeon James Parkinson who in 1817 published his classic monograph about six cases that all show similar symptoms, which he termed "*shaking palsy"* and now, two centuries later, the disease is named after its discoverer (Parkinson, 2002). He noted that all cases had in common: involuntary muscle movements, reduction of muscle tension, and postural instability whilst (he claimed) showing no signs of cognitive dysfunction (Parkinson, 2002). One of the key symptoms Parkinson recognised was tremor at rest. In later stages of the disease patients appeared to suffer from additional sleep disturbances, difficulties in walking and swallowing, and agitation (Forno, 1996; Parkinson, 2002). An important key factor that distinguished this form of palsy from others was the tremor that occurred at rest when the patient did not execute any voluntary movements. According to the international classifications of diseases (ICD-10) published by the World Health Organisation, PD falls within the category of extrapyramidal and movement disorders (G20), which encompasses patients with hemiparkinsonism, idiopathic parkinsonism, paralysis agitans, Parkinson's disease NOS (not otherwise specified), and primary Parkinson's disease (WHO, 1992). PD affects 0.3% of the entire population and 1% of the over 60s. Although disease onset is seldom before the age of 50, there is a steep increase in prevalence after the age of 60 (Forno, 1996; de Lau & Breteler, 2006).

## 1.2 Economic considerations

In the United Kingdom each of the 109,806 patients which have been diagnosed with PD are estimated to produce annual costs of £13,804 and the cost for the 1.2 million patients across Europe is estimated to cost an average €13.933 billion (McCrone *et al.*, 2007; Lees *et al.*, 2008; Gustavsson *et al.*, 2011). Demographic changes predict an increase in the number of elderly people, and hence the number of PD patients. As health care costs are constantly rising it is imperative to find remedies that cure, halt, or slow down the progression of the disease as well as improve the patients' quality of live. The European Union has realised that investments have to be undertaken now and currently 339 programs are funded under the EU 7$^{th}$ framework programme (http://cordis.europa.eu). The Brain Repair Group at Cardiff University is involved in three of such multinational research networks: NeuroStemCell, TansEuro,

and REPLACES. These grant consortia aim to push forward on important issues to get cell replacement therapies into the clinic.

## 1.3 Pathology

PD is now defined as a progressive, neurodegenerative disorder, in which the underlying pathology consists, among others, of a prominent degeneration of the pigmented, dopaminergic neurons in the substantia nigra pars compacta (SNpc), a brain structure in the ventral tegmental region of the brain (Olanow & Tatton, 1999; Lees *et al.*, 2008). Alongside to the overt loss of DAergic cells in the SN, histology of patients' brains has revealed fibrilisation of the protein α-synuclein throughout the brain, which leads to the formation of intracellular inclusions called Lewy bodies and Lewy neurites (Spillantini *et al.*, 1997; Spillantini *et al.*, 1998; Spillantini, 1999). Lewy bodies are of globular shape and mainly found in the cell perikarya whereas the Lewy neurites are spindle-like filaments and granular material located in the cellular processes (Braak *et al.*, 2003).

The motor impairment in PD is attributed to a dysfunction and degeneration in the nigro-striatal DA pathway. In the healthy brain DAergic neurons from the SNpc send afferents to the caudate-putamen (striatum) which is important, within the basal ganglia, for controlling voluntary movement, action selection, and stimulus-action associations (Hauber, 1998; Hayes *et al.*, 1998; Blandini *et al.*, 2000; Packard & Knowlton, 2002; Nicola, 2007; Rang *et al.*, 2007). Although other neurotransmitter systems, such as the serotonergic (5-HT), the acetylcholinergic (ACh), and the (nor-) adrenergic systems are affected as well, the primary neuropathology of PD is the degeneration of the neurons in the SNpc causing the depletion of striatal DA levels.

The Russian Konstantin Tretiakoff was the first who described in his doctoral thesis (1919) the loss of pigmented cells ('black stuff') in the SN togther with swelling of the remaining cell bodies in 54 brains of PD patients (Lees *et al.*, 2008). Carlsson and colleagues found that 3,4-dihydroxyphenylanine (a DA precursor) was able to reverse akinetic effects of reserpine-treated rabbits and mice (Carlsson *et al.*, 1957). Ehringer and Hornykiewicz were the first to report in 1960 the relation between the loss of DA in the striatum in patients diagnosed with idiopathic PD and postencephalitic PD (Ehringer & Hornykiewicz, 1998). Although they proposed the location of the DAergic cell bodies to reside within the striatum (large multipolar neurons), their finding was of utmost importance for establishing the hypothesis that

DA is not just a precursor of noradrenaline but a neurotransmitter in its own right, and, furthermore, that it is the loss of DA in the striatum that causes the akinetic symptoms seen in PD (Ehringer & Hornykiewicz, 1998). It has been shown subsequently by the use of the new flourecent method that the DAergic cell bodies actually reside in the SN and that they are projecting and innervating the striatum (Dahlstrom & Fuxe, 1964). In addition to the loss of DA neurons, protein aggregations (Lewy bodies) can be found within the brain of patients (Rinne *et al.*, 1989; Rinne, 1991; 1993; Spillantini *et al.*, 1997; Spillantini *et al.*, 1998; Harding & Halliday, 2001). The cytoplasmic accumulation of the misfolded protein α-synuclein has been linked to cell death via apoptosis in PD, although the exact mechanism remains elusive. Transgenic animal models which overexpress α-synuclein have been developed and were useful for studying the effect of agents that prevent/inhibit the misfolding of the protein (Lotharius & Brundin, 2002a; b; Soto, 2003; Buchman & Ninkina, 2008; Chesselet, 2008). In the human brain, Lewy bodies have been located throughout the brain of PD patients and the spread of these protein aggregations in PD is suggested to follow a defined pattern (Braak *et al.*, 2003; Braak *et al.*, 2005; Braak *et al.*, 2006). The pathology is first observed in the olfactory tubercle and the dorsal motor nucleus and then is seen to spread to include the ventral medulla, raphe nucleus, locus coeruleus and the SNpc. From there the pathology spreads further to the nucleus basalis of Meynert and amygdala and subsequently affects the cerebral cortex (Braak *et al.*, 2003; Braak *et al.*, 2005; Braak *et al.*, 2006). For a detailed description of pathology spread the reader is referred to Braak *et al.* (2003).

## 1.4 Symptomatology

PD is primarily considered a movement disorder with the following hallmark symptoms: akinesia (inability to initiate movement), bradykinesia (slowness in the execution of movement), hypokinesis (diminished or reduced movement), postural instability, muscular rigidity, and resting tremor (4-6 Hz) (Olanow & Tatton, 1999; Deumens *et al.*, 2002; Parkinson, 2002; Dauer & Przedborski, 2003; Bove *et al.*, 2005; Rang *et al.*, 2007). Common comorbidity with PD is depression, dementia (poor concentration, general slowness of cognitive speed, memory loss), hallucinations and psychosis (most related to high doses of levodopa), and sleep disturbances, as well as anxiety, impulsivity and fatigue have been reported (Shulman *et al.*, 2001; Racette *et al.*, 2002; McDonald *et al.*, 2003; Leibson *et al.*, 2006; Bertram & Williams, 2012; Latoo *et al.*, 2012). As the disease is progressive, symptoms usually worsen with time. Whilst at the onset of the disease many

symptoms can present themselves unilateral, at later stages of the disease the entire body is affected (Frank *et al.*, 2006). Dementia, autonomic dysfunctions, postural instability and heightened mortality risk are common in advanced PD (Olanow & Tatton, 1999; de Lau *et al.*, 2005).

Diagnosis of PD is difficult for the physician as a defitive confirmation can only be established post-mortem. As the disease is progressive the symptoms are small in the beginning and can easily be missed if not searched for. Next to the classical tremor, rigidity, akinesia, and postural instability, clinicians can notice micrography, changes in gait, facial expression, and loss of olfactory function (Frank *et al.*, 2006). Although brain imaging does not provide conclusive evidence, newly developed techniques as DAT-imaging can help diagnostics (Scherfler *et al.*, 2007; Felicio *et al.*, 2009; Brooks, 2010) as well as a trial period on levodopa-carbidopa for several weeks might be considered (Frank *et al.*, 2006).

## 1.5 Etiology

The etiology of PD is largely unknown but genetic and environmental factors do play a role. About 90% of the cases are idiopathic, while the remaining 10% are ascribed to inherited genetic mutation ('familial PD'), in which the disorder is thought to be manifest due to underlying genetic predispositions and/or environmental factors. Specific risk factors for developing PD include exposure to pesticides, herbicides, industrial chemicals, farming, living in a rural environment, as well es exposure to exogeneous toxins as trace metals, cyanide, and organic solvents (Olanow & Tatton, 1999). Several genes have been identified that are involved in the development or heightened risk of developing PD (dominant or recessive), which are α-synuclein, Parkin (PARK2), UCHL-1 (PARK5), DJ-1 (PARK7), PINK1 (PARK6), LRRK2 (PARK8), NR4A2 (NURR1), PARK3, PARK4, PARK9, PARK10 and PARK11 (de Lau & Breteler, 2006). Importantly, certain genetic abnormalities do heighten the risk for PD (Olanow & Tatton, 1999; de Lau & Breteler, 2006) but disease development is less certain as in other degenerative disease as Huntington's disease (HD). Most frequently herbicides and pesticides are associated with a higher risk of developing PD. Rotenone is one commonly used pesticide and exposure has been linked with the formation of Lewy bodies and some parkinsonian symptoms (Tanner *et al.*, 2001; Tanner *et al.*, 2009). In the more common idiopathic form of PD, the progression of the disease is relatively slow. The first overt motor abnormalities are usually detected after approximately 80% of the nigral neurons are lost and the striatal DA levels are

depleted to 60% - 80% of the original levels (Berhnheimer *et al.*, 1973; Price *et al.*, 1978; Mardsen, 1983; Uhl *et al.*, 1985; Uhl *et al.*, 1994; Rang *et al.*, 2007).

## 1.6 Cognitive symptoms in PD

Traditionally research has focused on the more overt motor symptoms of the disease. Recently research has shown that PD patients also exhibit cognitive and psychiatric impairments. These cognitive impairments may even precede the first overt motor symptoms. A considerable subset of patients report episodes of depression, which can be as disabling as the motor impairments because they can reduce the patients' quality of life (Kuopio *et al.*, 2000). Non-motor symptoms include neuropsychiatric symptoms (depression, apathy, anxiety, anhedonia, attention deficit, hallucinations, dementia, and panic attacks), sleep disorders (restless leg, REM sleep behaviour disorder, non-REM sleep-related movement disorder, daytime somnolence, vivid dreaming, insomnia, sleep-disordered breathing), autonomic symptoms (bladder disturbances, sweating, orthostatic hypotension, sexual dysfunction, dry eyes), gastrointestinal symptoms (dribbling of saliva, ageusia, dysphagia, reflux, nausea, constipation, faecal incontinence), sensory symptoms (pain, paraesthesia, olfactory disturbance), as well as fatigue, diplopia, blurred vision and weight loss (Chaudhuri *et al.*, 2006). The most prominent cognitive impairments in patients, present as disturbances in memory, attention, executive function, and visuospatial function (Aarsland *et al.*, 2010). Furthermore, PD patients in early stages of the disease have been shown to perform worse on several cognitive/neuropsychological tests when compared to age-matched healthy controls (Lees & Smith, 1983; van Spaendonck *et al.*, 1998; Cools *et al.*, 2010). Performance on the Wisconsin Card Sorting Task revealed that PD patients produce more perseverative errors and show difficulties in switching between response sets, although they do not show an impairment in their general intelligence score as determined by the Wechsler Adult Intelligence Scale (Lees & Smith, 1983). Taken together, although the predominant disabling nature of the disease is mainly linked to the motor component, the non-motor symptoms appear to impact more greatly on impairing the quality of patient life (Chaudhuri *et al.*, 2006; Aarsland *et al.*, 2010).

## 1.7 The basal ganglia and the striatum

The human striatum consists of two nuclei, the caudate nucleus and the putamen, which are separated by the internal capsule. The striatum is considered to be part of a larger set of nuclei which together make up the basal ganglia: comprising the striatum, the internal and external segment of the globus pallidus (GPi and GPe), the subthalamic nucleus (STN), and the substantia nigra pars reticula (SNpr) and pars compacta (SNpc). The striatum is the main input structure of the basal ganglia whereas the GP and the SNpr are the output nuclei. The striatum has four major inputs, which are topographically organized from the cortex and the thalamus (glutamatergic) and more diffusely from highly branched brain stem regions (DAergic and serotonergic). The neurons in the GPe/GPi, STN and SN display autonomous pacemaker activity, i.e. always providing a tonic level of activation/suppression, depending on their respective neurotransmitter (Gerfen, 1984; 1985; Joel & Weiner, 1994; 1997; Bolam *et al.*, 2000; Joel & Weiner, 2000; Gerfen & Surmeier, 2011; Mathai & Smith, 2011). The output of striatal information processing terminates ultimately in the thalamus where activation leads to a facilitation of movement, whereas an inhibition has the opposite effect.

The basal ganglia are a functionally heterogeneous group of nuclei through which pass several distinct but parallel interconnected pathways or loops of connections, named after the functional associations of the particular cortical areas from which each loop originates (Alexander *et al.*, 1986; Alexander & Crutcher, 1990; Alexander *et al.*, 1990; Wichmann & DeLong, 1996). Thus, each 'cortico-striatal' loop arises from a different part of the cortical mantle and projects to topographically different areas within the striatum, the GP, and the thalamus, where information is processed and eventually fed back to the cortical areas of origin as well as exhibiting a degree of convergence on cortical and subcortical motor outputs. Although the loops are thought to run in parallel, there is considerable overlap as well as exchange of information between the individual loops. The motor loop (control of facial, limb and trunk muscles), for example runs from the cortex to the supplementary motor area, to the putamen, and further to the GPi/SNr and ends in the ventral-lateral thalamus from which information is fed back into the cortex (Alexander & Crutcher, 1990; Alexander *et al.*, 1990). Other proposed loops are the oculomotor loop (controls saccadic eye movements), the dorsolateral-prefrontal loop (exectutive functions), the lateral orbitofrontal loop and the anterior-cingulate loop (emotions) (Alexander *et al.*, 1986).

The output projections of the striatum have been divided into a direct and an indirect pathway. The direct pathway projects from the striatum to the GPi and SNpr and from there to the thalamus. The indirect pathway projects from the striatum to the GPe, which in turn projects to the GPi and the SNpr, which further terminates in the thalamus. A second projection from the GPe projects to the STN which then projects to the GPi and the SNpr (see Figure 1.1 and Figure 1.2). Although nowadays the clear cut distinction has been questioned as there is some overlap between the pathways, it still remains a good heuristic on a conceptual level (Mathai & Smith, 2011).

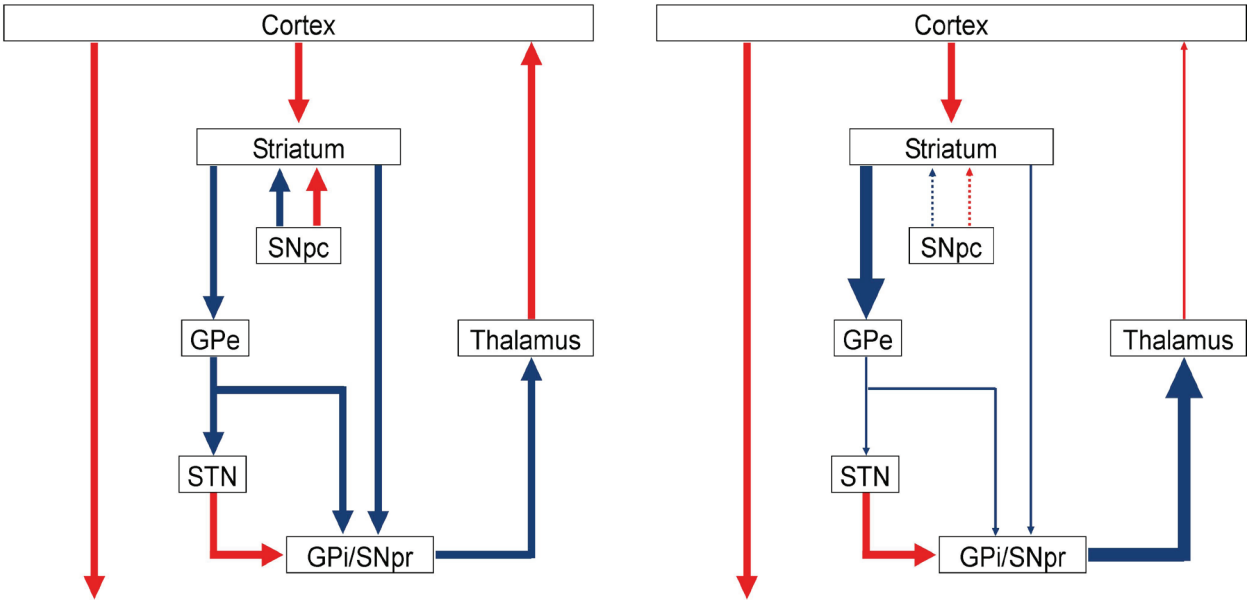


**Figure 1.1**. Schematic representation of the circuitry of the basal ganglia motor loop in the normal (left side panel) and parkinsonian (right side panel) brain. Adapted from Wichmann and DeLong (1996). Abbreviations: SNpc = substantia nigra pars compacta; SNpr = substantia nigra pars reticula; STN = subthalamic nucleus; GPi = globus pallidus internal segment; GPe = globus pallidus external segment. Red arrows represent excitatory connections; blue arrows represent inhibitory connections. Note that in the parkinsonian state the net output of the GPi/SNpr results in a suppression of the pacemaker activity of the thalamus.

The basal ganglia circuitry mainly uses γ-amino-butyric acid (GABA) as the inhibitory neurotransmitter and glutamate as the excitatory neurotransmitter. From the cortex excitatory glutamatergic connections terminate in the STN and in the striatum together with DAergic projections from the SNpc. The striatum uses GABA and substance P as inhibitory neurotransmitters along the direct pathway from the striatum to the GPi/SNpc in contrast to GABA and enkephalin in the indirect pathway to inhibit GPe. There are two main classes of DAergic receptors in the striatum ($D_1$ and $D_2$ type) via which this structure can be excited ($D_1$ receptors) or inhibited ($D_2$

receptors), with a bias to $D_1$ influence over direct pathway projections and $D_2$ influence over indirect pathway projections (Yung *et al.*, 1995; Yung *et al.*, 1996). From the STN excitatory glutamatergic projections target in the GPi/SNpr. The output from the latter structure to the thalamus is inhibitory and GABAergic (Alexander & Crutcher, 1990; Wichmann & DeLong, 1996; Hauber, 1998). The direct and indirect pathways can be distinguished by their neurotransmitter expression (direct pathway: Dynorpnin, Substance P; indirect pathway: Enkephaline) as well as by relative expression of $D_1$ and $D_2$ like receptors, respectively (Gerfen & Surmeier, 2011). The direct pathway sends its outputs to the GPi and the SNpr, which both forward the information to the thalamus, whereas projections from the indirect pathway mainly innervate the GPe (Crittenden & Graybiel, 2011). This striatal circuitry comprising direct and indirect pathway allows for different degrees of inhibitory effects on the thalamus. If the normal balance is disturbed as in PD or HD, the thalamus can receive too much inhibition due to an overactive indirect pathway (PD), resulting in inhibition of movement as seen in PD, or it can lead to the opposite, a facilitation of movement due to too little inhibition of the thalamus as in HD (Wichmann & DeLong, 1996). Although the direct/indirect pathway model is an oversimplification of the actual anatomy of the human basal ganglia with its parallel and overlapping loops the model has great explanatory value (Mathai & Smith, 2011).

The neurons of the striatum are principally classified by size and morphology and 90-95% of striatal neurons are termed medium and spiny neurons (MSNs) which can be further classified into four subgroups, depending on their co-expression of GABA with other neurotransmitters. One type is associated with parvalbumin and $D_2$ receptors, whereas another type coexpresses calretinin. Type three coexpresses somatostatin, neuropeptide Y, NOS, and $D_2$ receptors, whereas type four coexpresses choline acetyltransferase (ChAT) and dopamine- and cAMP-regulated neuronal phosphoprotein) DARPP-32 (Hirsch *et al.*, 1989; Holt *et al.*, 1997; Crittenden & Graybiel, 2011).

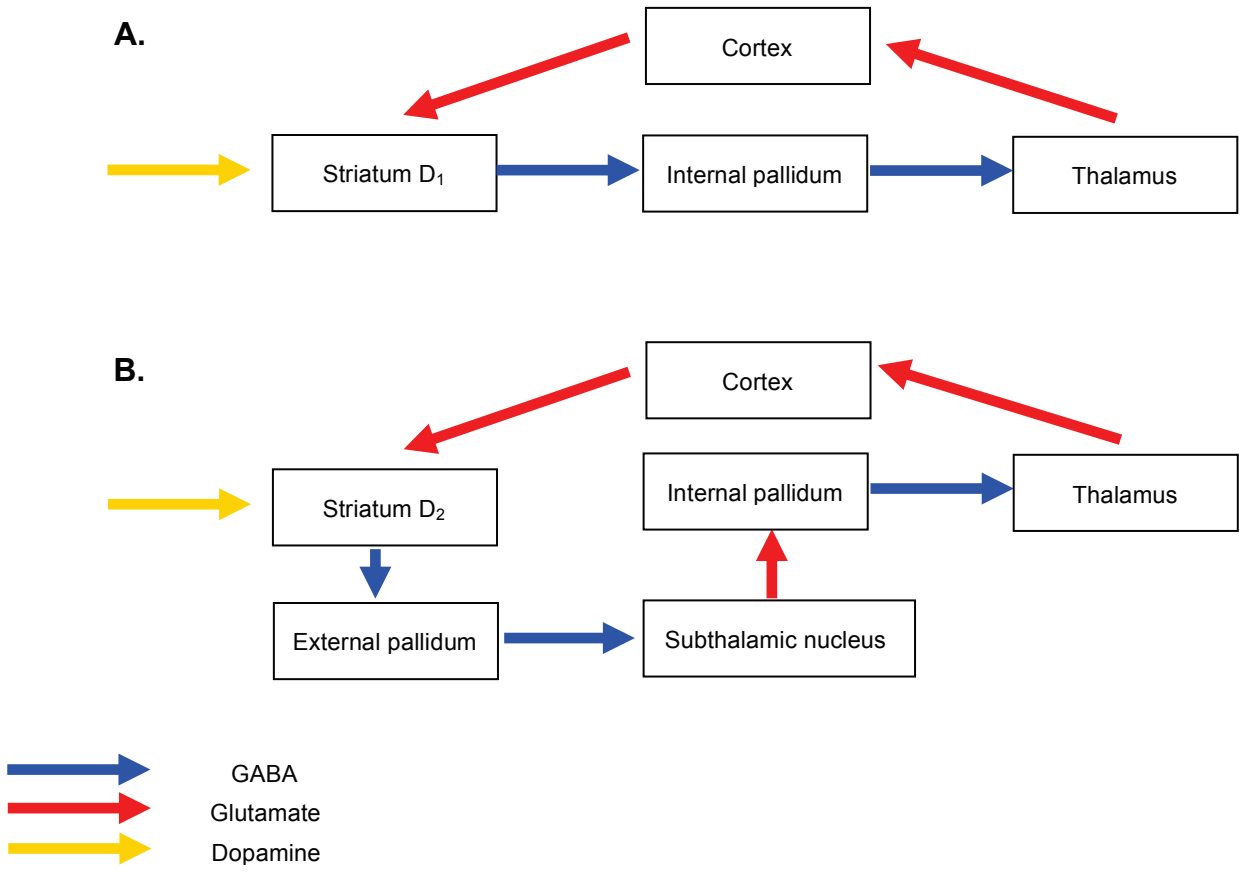


**Figure 1.2.** Schematic representation of the direct (**A.**) and the indirect (**B.**) pathways of the basal ganglia (adapted from Graybiel, 2001).

Another distinction of striatal organisation can be made by their neurotransmitter staining for acetylcholinesterase (AChE) and opiate-receptors, which have revealed darker stained "patches" or "striosomes" and matrix regions with lighter or a lack of staining within the striatum. Patches are generally defined by their high opiate receptor binding whereas matrix cells are staining for AChE (Gerfen, 1984; 1985; Gerfen *et al.*, 1985; Gerfen *et al.*, 1987; Nastuk & Graybiel, 1988; Hirsch *et al.*, 1989; Jimenez-Castellanos & Graybiel, 1989). Neurons within the matrix mainly project to associative and sensorimotor regions of the cortex, whereas those of the patch regions project to the limbic system and are the only region in the striatum that contains cells that possess direct synapses with DAergic cells in the SNc (Holt *et al.*, 1997; Crittenden & Graybiel, 2011). The patches are densely innervated by projections from orbitofrontal, anterior cingulated, insular cortex whereas matrix MSNs are mainly innervated by terminals that originate from somatosensory, motor and association cortices (Crittenden & Graybiel, 2011). Both, patches and matrix, receive DAergic input from the SNpc, although from different regions. The ventral tier of the A9 cell group predominantly projects to patches, whereas cells from the dorsal tier of the SNc, as well as cells from the retro-rubal area (A8) and VTA (A10)

innervate the matrix (Gerfen *et al.*, 1985; Gerfen *et al.*, 1987; Joel & Weiner, 2000). DA in the striatum is not only involved in the facilitation of movement, but also it has been implicated in decision-making processes. Although DAergic cells in the SNpc display pacemaker like activity patterns and provide tonic DA levels in the striatum, when an animal encounters a positive reward, DA release is temporarily increased whereas it is temporarily decreased in aversive events. These findings have linked the phasic DA signal to be attributed to the concepts of promoting long-term depression and long-term potentiation, the underlying mechanisms of learning (Gerfen & Surmeier, 2011).

## 1.8 Pharmacological treatment of PD

None of the pharmacological compounds tested so far have been able to halt, slow, or reverse the progression of the disease, as also with all other therapeutical approaches. The majority of compounds available are aimed at increasing the availability of DA in the brain, thereby restoring the neurotransmitter that is lost during disease progression. DA is synthesized from L-tyrosine via the rate limiting enzyme tyrosine-hydroxylase to L-Dihydroxyphenalaline (l-DOPA, Figure 1.3). Via DOPA decarboxylase and Aromatic L-amino acid decarboxylase, l-DOPA is then transformed to DA. DA can be further synthesised to noradrenaline via DA-β-hydroxylase and from there via Phenylethanolamine *N*-methyltransferase to adrenaline. The most successful line of research has been in restoring the levels of DA in the brain by administration of levodopa (l-DOPA), a DA precursor that crosses the blood brain barrier and can be converted to DA by the remaining neurons. Metabolic products of DA degradation via monoamine oxidase (MAO) and catechol-*O*-methyl transferase (COMT) are homovanillic acid (HVA) and 3,4-Dihydroxyphenylacetic acid (DOPAC). DA that is not broken down is taken back into the synapse and stored in vesicles via the vesicular monoamine transporter (VMAT)-2.

Reserpine is a drug that inhibits the VMAT-mediated transport of DA (and others) into the DAergic cell for packaging into the vesicles, thereby causing DA deficiency. Initial studies showed that the behaviour of DA depleted mice and rabbits, via injection of reserpine, could almost completely be restored by injections of l-DOPA (Carlsson *et al.*, 1957; Hornykiewicz, 2001; 2010). Since then much progress has been made and l-DOPA is currently the standard therapy for PD patients (Schapira, 2005; Hauser, 2009; Schapira *et al.*, 2009). Orally taken l-DOPA is broken down in the gut and liver

relatively fast if not taken in combination with an inhibitor of peripheral decarboxylase (i.e. carbidopa) to prevent breakdown and ensure availability in the brain (Schapira, 2005; Hauser, 2009; Nagatsua & Sawadab, 2009; Schapira *et al.*, 2009). Additional medications that provide some symptom relief include MAO and COMT inhibitors which slow the breakdown of DA, as well as DA agonists (Chen *et al.*, 2007; Caslake *et al.*, 2009; Schapira *et al.*, 2009).

Although l-DOPA provides great relief for several years its effectiveness is limited to the remaining DA cells and tolerisation of the receptors. The effective window is usually 5-10 years and, with progression of the disease higher doses of the drug have to be taken. Side effects of l-DOPA ingestion include gastro-intestinal abnormalities, orthostatic hypotension ('head rush/dizziness'), hallucinations, sleepiness and behavioural problems (Schapira *et al.*, 2009). The main motor side effects that are reported are severe motor disability and rigidity that occurs in the "off-phase" of the drug and dyskinesias (jerky involuntary movements) that can occur at higher doses (Marsden & Parkes, 1976; Olanow *et al.*, 2004; Santini *et al.*, 2007; Lane & Dunnett, 2008; Santini *et al.*, 2008; Hauser, 2009; Santini *et al.*, 2009; Lane & Smith, 2010; Stocchi *et al.*, 2010). Therefore, although l-DOPA therapy is highly effective, especially during early stages of PD, the effects are limited to a narrow therapeutic window and higher doses are less well tolerated (Bjorklund *et al.*, 2003; Olanow *et al.*, 2004; Schapira, 2005; Schapira *et al.*, 2009). Alternative strategies, like cell replacement therapies offer promising alternatives to circumvent these shortcomings.

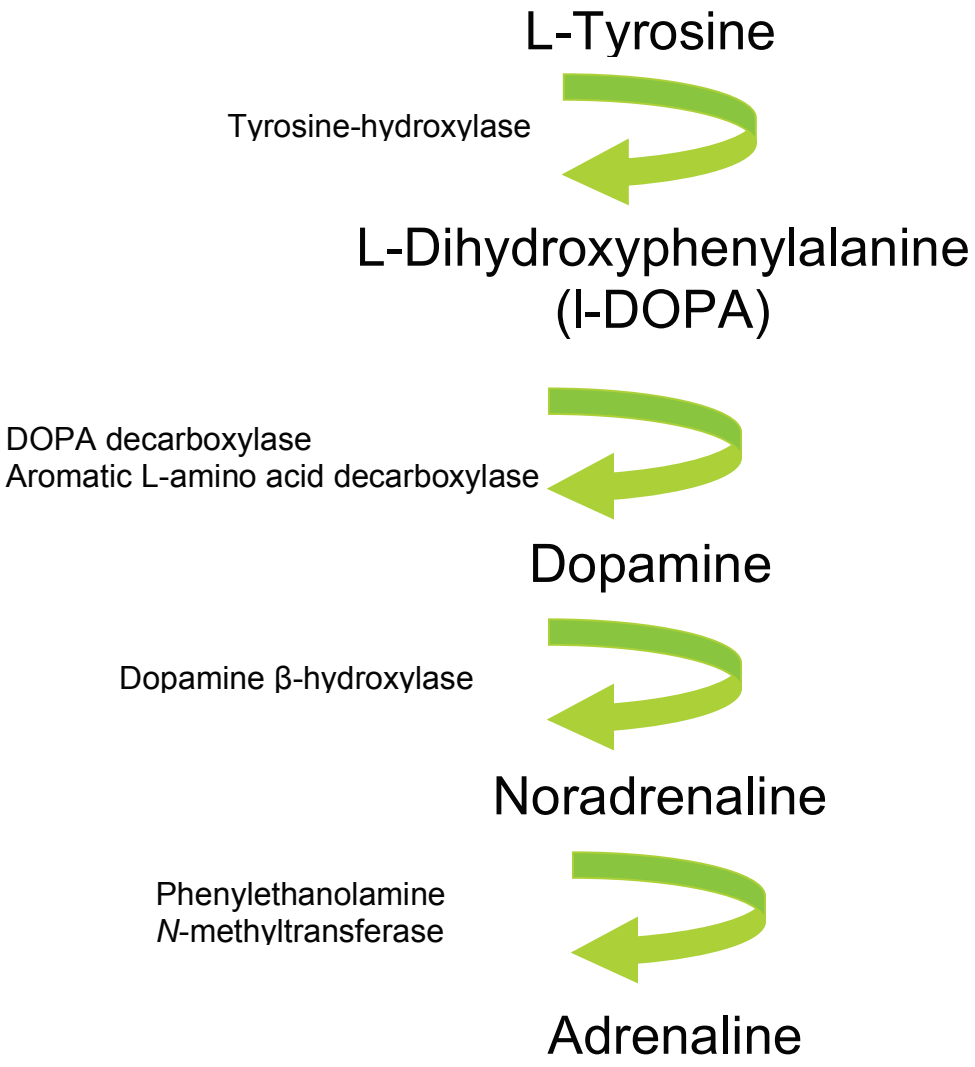


**Figure 1.3**. Synthesis of catecholamines.

## 1.9 Deep brain stimulation

Another promising approach to alleviate the motor symptoms is deep brain stimulation (DBS). In DBS, electrodes are implanted into the patient's brain, usually targeting the subthalamic nucleus (STN) and the electrodes are connected to a pacemaker which is transplanted in the chest delivering an electric current through the electrodes (Benabid *et al.*, 1991; Benabid *et al.*, 1993; Phillips & Brown, 1999; Blandini *et al.*, 2000; Temel *et al.*, 2005; Temel *et al.*, 2006a). DBS treatment is effective in controlling the motor symptoms and suited for advanced stages of PD when conventional therapies such as l-DOPA medication are no longer effective. Major drawbacks of DBS are the invasive surgery and the risk of behavioural side effects. For example, patients in on-states (undergoing stimulation) have been

reported to develop manic personality and changes in character (Leentjens *et al.*, 2004).

## 1.10 Transplantation as a therapy for Parkinson's disease

As mentioned, l-DOPA relies on the remaining cells to convert this precursor to DA in the brain, and, therefore it has only a limited therapeutic window and higher doses produce disabling dyskinesias (Bjorklund *et al.*, 2003; Olanow *et al.*, 2004; Schapira, 2005; Schapira *et al.*, 2009). Transplantation of DAergic cells is therefore a promising alternative for patients who suffer from drug-induced dyskinesisas (Bjorklund *et al.*, 2003; Winkler *et al.*, 2005). The underlying principle of transplantation is that DAergic precursors are harvested from embryos and then transplanted into the patient's brain. Within the host brain, the precursors develop into DAergic neurons replacing the lost neurons and form connections with the remaining striatal cells.

Transplantation of DAergic cells as a therapy for PD is based on two major assumptions: (i) the hallmark symptoms of the disease are based on degeneration of the DAergic cells in the nigro-striatal pathway, and (ii) engrafted cells will survive the transplantation procedure and develop into mature DA releasing cells that release DA into the depleted striatum, thereby alleviating (at least in part) the symptoms caused by the loss of DA (Bjorklund *et al.*, 2003).

Proof that cell replacement is a viable and highly promising therapy for PD comes from numerous experiments in lesioned rats, mice and non-human primates. Early studies have shown that engrafting unilateral 6-OHDA lesioned rats with DA rich tissue grafts resulted in a reduction in some tests of motor behaviour, specifically, drug-induced rotations in response to amphetamine and apomorphine which correlated highly with the amount of DA re-innervation of the depleted striatum (Perlow *et al.*, 1979; Dunnett *et al.*, 1981a; Dunnett *et al.*, 1984; Torres & Dunnett, 2007). Initial studies engrafted tissue pieces into cortical cavities that were above the striatum (Bjorklund & Stenevi, 1979; Dunnett *et al.*, 1981a; b). Later studies looked at transplantation into the denervated tissue by employing enzymatic and mechanical dissociation of the cells into a quasi-single cell suspension which allowed delivery of the cell suspension via stereotaxic surgery into the lesioned striatum (Schmidt *et al.*, 1981; Bjorklund *et al.*, 1983a; b; Dunnett *et al.*, 1983b; a; Schmidt *et al.*, 1983; Dunnett *et al.*, 1984). Alongside transplanting cells as a suspension graft, rather than solid tissue pieces, there are three main factors that affect graft survival in the adult

brain, which are (i) age of donor, (ii) site of transplantation, and (iii) technical factors (Stenevi & Bjorklund, 1978). Survival of fetal tissue is better than tissue derived from the adult or post-natal brain (Stenevi & Bjorklund, 1978). However, even with prenatal ages, the right developmental time point for transplantation has to be found. It has been shown that engrafting cells just before their final division has been found to greatly enhance cell survival, leading to larger grafts (Fricker *et al.*, 1997a; Fricker *et al.*, 1997b; Gates *et al.*, 2006; Torres *et al.*, 2008a; Torres *et al.*, 2008b; Bye *et al.*, 2012). Furthermore, the site of transplantation has a great influence on cell survival. Cells survive better when they become quickly re-vascularised (Stenevi *et al.*, 1976; Stenevi & Bjorklund, 1978). Furthermore, sites which are thought to be (at least partly) immuno-privileged, such as the anterior eye-chamber or the brain, allow for better survival (Malmfors & Olson, 1967; Olson & Malmfors, 1970; Dunnett, 2010). Technical factors to increase graft survival range from the use of aseptic surgery, to how the tissue is transplanted into the brain. It has been shown that the use of smaller deposits placed using fine glass cannulae leads to better survival than metal cannulae (Stenevi *et al.*, 1976; Nikkhah *et al.*, 1993; Nikkhah *et al.*, 1994a; Nikkhah *et al.*, 1994b; Nikkhah *et al.*, 1994c; Nikkhah *et al.*, 1995; Nikkhah *et al.*, 2009).

Unilateral or bilateral lesioned/transplanted animals displayed recovery on some, but not all, tests of sensori-motor function (Dunnett *et al.*, 1981b; a). Tests that require a higher degree of circuitry reconnection are more difficult to restore. The limited functional recovery that has been reported in animal studies of PD has been ascribed to incomplete reconstruction of the nigro-striatal circuit. In the majority of studies, including animal and human clinical trials, the engrafted cells are transplanted into the striatum, the target area of the DAergic nerve terminals, as opposed to the SN where the cell bodies originate. The main reason for this ectopic graft placement had been the better re-innervation found with this model (Bjorklund *et al.*, 2003). Furthermore, several reports have questioned the ability of striatal grafts to grow the long distance from the SN to the lesioned striatum (Barker & Dunnett, 1999). It has been argued that one reason why reconnection from SN to the striatum is not achieved routinely is the glial scar/extent of neuronal degeneration that is achieved by the complete lesion models, which was necessary until recently to distinguish donor from host fibres (Aguayo *et al.*, 1984; Doucet *et al.*, 1990; Dunnett, 2010). It has been speculated that using lesions to the SN there would be enough fibres left to guide the axonal outgrowth into their target areas, and indeed, using Green Flourecent Protein (GFP), it has been reported that cells transplanted into the SN do grow projections that re-innervate the striatum, nucleus accumbens, and even the

frontal cortex in a mouse model of PD (Thompson *et al.*, 2005; Thompson *et al.*, 2009). It has been speculated that ectopic graft placement does not achieve true "circuit reconstruction" because the DAergic cells are deprived of their natural input/environment and DA release might not be as regulated as it would have been if cells would reside in their normal habitat (Winkler *et al.*, 2000; Bjorklund *et al.*, 2003). Nevertheless, nigral fetal cells that are transplanted into the rat striatum do survive, form synaptic contacts and release DA into the surrounding tissue (Freund *et al.*, 1985; Doucet *et al.*, 1989; Fisher *et al.*, 1990; Wictorin *et al.*, 1990b; Wictorin *et al.*, 1992).

After the initial reports that nigral fetal rat tissue was able to survive, restore DA levels in the striatum and alleviate motor symptoms in the adult rat brain, the first clinical trials were planned. The first trials were conducted using autologous chromaffin cells of the adrenal medulla as these cells were known to produce catecholamines and reverse motor symptoms in the rat (Freed *et al.*, 1980; Freed *et al.*, 1981; Backlund *et al.*, 1985; Freed *et al.*, 1990; Date, 1996; Dunnett, 2010). Unfortunately, clinical effects were small although catecholaminergic metabolites were elevated after the graft surgery (Backlund *et al.*, 1985). A follow-up trial in Mexico reported a dramatic improvement in patients rigidity and akinesia and a reduction of tremor (Madrazo *et al.*, 1987). This sparked a series of studies, but the results could not replicate the dramatic improvement previously reported (Bakay *et al.*, 1990; Goetz *et al.*, 1991). In contrast, the publication of a multicentre study in North America revealed that 18% of these patients died, and 50% of the deaths were directly related to the surgical procedure. Although some beneficial effects were reported up to two years post surgery as determined via the UPDRS, other measures did not improve. Additional adverse effects reported were persistent psychiatric morbidity (Goetz *et al.*, 1991). The results led investigators to move on from medullary grafts to the more promising alternative of fetal nigral tissue. Several small open label clinical trials have shown that embryonic fetal tissue that is transplanted directly into the brain of PD patients can survive and show significant and promising functional benefits on neurological assessment scales (Lindvall *et al.*, 1988; Madrazo *et al.*, 1988; Lindvall *et al.*, 1989; Lindvall *et al.*, 1990a; Lindvall *et al.*, 1990b; Lindvall *et al.*, 1994). Engrafted DAergic cells into the putamen have been shown, via use of the agent [$^{11}$C]-raclopride ($D_2$ receptor antagonist), to (i) release DA from the synapses, (ii) lower UPDRS scores two years post-transplantation, (iii) reduced time off medication from 1 year post engraftment onwards and (iv) to increase [$^{18}$F]-dopa uptake in the treated hemisphere (Piccini *et al.*, 1999).

It has been suggested that patient selection is essential for improved therapeutic outcome of DA cell replacement therapy. Younger patients showed greater benefit from the transplants than older patients (Freed *et al.*, 2001; Bjorklund *et al.*, 2003). Other factors considered before surgery were baseline responsiveness to l-DOPA, severity of PD symptoms at time of surgery, tailoring of graft location, age of donor tissue (5.5 – 9.0 weeks gestation), number of embryos used (2-9 per patient), hibernation of the tissue before use (up to 4 weeks), tissue preparation (cell suspensions vs. tissue pieces), and immunosupression method (Freed *et al.*, 2001; Winkler *et al.*, 2005).

The first double-blind, sham surgery-controlled trial disclosed showed that the benefits obtained by the engraftment of embryonic DA rich tissue into the caudate-putamen of patients with advanced PD showed little clinical benefit as assessed by the UPDRS, the Schwab and England scale, and the scale of the Core Assessment Program for Intracerebral Transplantations (Freed *et al.*, 2001). Next to the marginal clinical improvements, there were severe side effects such as graft-induced dyskinesias reported in several patients (15%), even during off medication. Division into sub-groups showed that patients that were more likely to benefit from the cell replacement therapy were younger (<60 years) although individuals of the older group (>60 years) also showed some improvements (Freed *et al.*, 2001). Subsequent inspection of the data has shown that this effect was most likely due to responsiveness to l-DOPA rather than age of the patient *per se* (Bjorklund *et al.*, 2003; Winkler *et al.*, 2005). The improvements in the neurological rating scales were supported by increased [$^{18}$F]-DOPA uptake and survival of TH-ir cells in 2 deceased patients (Freed *et al.*, 2001).

The report sparked a series of replies and discussions into (i) why the reported therapeutic effect was low overall, especially compared to some of the improvements seen in the earlier open-label clinical trials, and, (ii) why the side effects were so high (Dunnett *et al.*, 2001; Bjorklund *et al.*, 2003; Winkler *et al.*, 2005; Brundin *et al.*, 2010). Freed et al. (2001) reported that cell survival was relatively low, compared to previous reports using open label clinical trials (Brundin *et al.*, 2001). Furthermore, the Freed study was criticised for being variable and inconsistent with previous reports in terms of tissue preparation, implantation techniques and immunosupression (Brundin *et al.*, 2001; Dunnett *et al.*, 2001). Major differences to

previous reports were the relatively low number of TH-ir cells transplanted (Kordower *et al.*, 1995; Bjorklund *et al.*, 2003).

There are several problems associated with the transplantation of cells derived from human fetal tissue. The largest constrains concern the ethics, availability, logistics and quality control that is associated with obtaining the optimal donor tissue (Bjorklund *et al.*, 2003). Alternative cell sources such as stem cells and/or xenogeic cells are being investigated as potential alternatives (Barker *et al.*, 1999; Barker *et al.*, 2000; Barker, 2002; Bjorklund *et al.*, 2003; Harrower *et al.*, 2006; Kriks *et al.*, 2011). Although promising in providing a solution to some of the above mentioned problems, using stem cells or xenografts harbour their own problems such as extensive proliferation and/or zoonotic infection (Barker *et al.*, 2000; Dunnett *et al.*, 2001; Bjorklund *et al.*, 2003).

A complementary experimental approach, which could be used in conjunction with cell replacement therapies, is to decrease the progression of the disease by delivering brain growth factors (Sinclair *et al.*, 1996; Kirik *et al.*, 2000a; Kirik *et al.*, 2000b; Kirik *et al.*, 2001a; Kirik *et al.*, 2004; Monville *et al.*, 2004; Dowd *et al.*, 2005b; Torres *et al.*, 2005a; b). As PD is a progressive neurodegenerative disorder once it is diagnosed the ideal would be to halt or slow down disease progression. Neuroprotection has been demonstrated in research models using trophic factors like GDNF or BDNF which can protect cells from oxidative stress caused by 6-OHDA lesions. Other approaches have used lazaroids or anti-apoptotic agents to promote cell survival (Brundin *et al.*, 2000b; Castilho *et al.*, 2000). In a phase I clinical trial the direct delivery of GDNF into the putamen of 5 patients has shown an increase in [$^{18}$F-]dopa influx and a decrease in scores on the Unified Parkinson's Disease Rating Scale (UPDRS) scores motor and daily living subscales (Gill *et al.*, 2003). Although the use of GDNF showed promising results in the 6-OHDA lesioned rat model of PD and in the open-lable clinical trial mentioned above, the first placebo-controlled clinical trial failed to show any significant benefits and the trial had been stopped prematurely because of adverse effect in the American arm (Lang *et al.*, 2006; Hutchinson *et al.*, 2007). A recent publication has shown that indeed GDNF was not effective in protecting DAergic cells from α-synuclein pathology (Decressac *et al.*, 2011).

In summary, cell replacement therapies have several advantages over current drug-based therapeutic approaches. The main advantage of transplantation of cells is that

it has the potential to restore DA levels in the brain on a sustainable basis, at physiological levels, under local regulation, and delivered synaptically to the precise areas where DA is lost (Dunnett *et al.*, 2001; Bjorklund & Dunnett, 2007b; Brundin *et al.*, 2010). Furthermore, as cell survival has been shown long term, it is not affected by a small therapeutic window as, e.g. l-DOPA therapy, which is dependent on remaining DA turnover in the brain (Dunnett *et al.*, 2001; Bjorklund & Dunnett, 2007b). Cell replacement therapy can be a promising treatment when done correctly. Graft-induced dyskinesias have been associated with DA-Hotspots in the brain due to the grafting technique (Winkler *et al.*, 2005; Brundin *et al.*, 2010). However, one of the main issues raised in cell transplantation is the small percentage of cells, specifically DAergic cells that survive the transplantation process (Barker *et al.*, 1996; Bjorklund *et al.*, 2003). Some progress has been made to increase the survival of donor tissue in animal models of the disease and it has been shown that the use of younger donor tissue or trophic factors can increase cell survival (Annett *et al.*, 1997; Torres *et al.*, 2005a; Torres *et al.*, 2007; Torres *et al.*, 2008a; Kauhausen *et al.*, 2013). Taken together, although cell replacement therapy is currently hampered by several factors to become a mainstream therapy as an alternative to DBS and pharmacological approaches (Bjorklund *et al.*, 2003), it still holds great promise. The problems of cell survival, storage, differentiation, etc. are currently addressed in European multi-centre trials and most importantly, via inspection of individual cases, clear proof-of-principle data shows that patients benefit long term from the approach. Selection of the right patient population will give us further insights as to whether cell replacement therapy is a viable alternative to the current therapeutic options. Therefore, although transplantation studies in humans have shown limited success, a subset of patients have shown to have benefited from the engrafting (Dunnett & Bjorklund, 1997; Brundin & Hagell, 2001; Fricker-Gates *et al.*, 2001; Fricker-Gates & Dunnett, 2002; Bjorklund *et al.*, 2003; Bjorklund & Dunnett, 2007b; Bjorklund *et al.*, 2009; Brundin *et al.*, 2010).

## 1.11 Dopaminergic cells

Since the first description of catecholaminergic cell groups in the brain (Carlsson *et al.*, 1962; Dahlstrom & Fuxe, 1964) and the advent of immunohistochemical techniques, nine major DA cell groups have been identified in the brain. Of importance for this work are the three DAergic cell groups A8 (retrorubal area), A9 (SN), and A10 (VTA), which are located in the mesencephalic region of the brain. In the rodent there are about 20,000 to 30,000 DAergic cells in mice, depending on

strain (Nelson *et al.*, 1996) and 1,300 (A8), 10,500 (A9) and 10,200 (A10) DAergic cells in rats (German & Manaye, 1993) distributed over all three cell groups. In humans, there are 400,000 to 600,000 DAergic cells in these three areas, with about 70% of cells being located in the SN (Bjorklund & Dunnett, 2007a). However, an absolute characterisation based on immunohistochemical methods is difficult as SN cells do co-express different markers complimentary to TH and their projections do not always innervate specific targets (about 5% of cells do send projections to both, patch and matrix striatal cells). Although there is a low level of overlap, the two major cell populations in the SN and VTA can be identified by their coexpression of either TH and the potassium channel subunit G protein activated inward rectifier potassium channel (GIRK)-2 for DA cells in the SN and TH and calbindin for DA cells in the VTA (Thompson *et al.*, 2005; Bye *et al.*, 2012). However, this distinction is not absolute as a large proportion of the DA cells in the respective nucleus does co-express the respective protein and can therefore be identified in combination with their cell morphology. SN cell morphology has been described as larger and more angular in shape, whereas VTA cells are smaller and rounder by comparison to the former cell type (Thompson *et al.*, 2005; Thompson *et al.*, 2009). The third factor by which they can be identified are their projection patterns. Whereas SN DA cells largely send out their efferents to the dorsal striatum, VTA cells largely project to the ventral striatum including the nucleus accumbens, parts of the limbic system, and further to the prefrontal cortex (Bjorklund & Dunnett, 2007a; Thompson *et al.*, 2009).

Other methods of distinguishing these cell types from other ventral mesencephalic cells include characterisation of their electrophysiology, since both sub-groups of DA cells can be characterised by their pacemaker activity and slow action potentials. SN and VTA cells share many electrophysiological properties and can only be distinguished on a cell population level (Karschin *et al.*, 1996; Neuhoff *et al.*, 2002). In recent reports it has been shown that when transplanting primary fetal ventral mesencephalon (VM), consisting of developing neurons from both SN and VTA, into 6-hydroxydopamine (6-OHDA)-lesioned rats, the transplanted cells show a different pattern of distribution within the graft and connection to the host brain (Thompson *et al.*, 2005). Cells in the centre of the graft are on average smaller in diameter (12.9 µm vs. 19.1 µm), and a larger proportion stained positive for calbindin whereas cells in the periphery of the graft were larger and a larger proportion was positive for GIRK-2 (Thompson *et al.*, 2005). It has been suggested that next to being DAergic, cells used for transplantation might need to be of the A9 phenotype (Lindvall & Bjorklund, 2004). Indeed, from retrograde tracing and behavioural assessment previous studies

report that (i) prelabeled cholera toxin subunit β injected into the dorsal striatum stained cells that were located in the periphery of the graft and many of the cells also co-labelled for TH and GIRK-2 and were larger and angular in shape, therefore suggesting that A9 type cells predominately re-innervate the surrounding striatal structure (Thompson *et al.*, 2005; Thompson *et al.*, 2009). In contrast, frontal cortical injections largely labelled cells in the centre of the graft which were round and calbindin positive (Thompson *et al.*, 2005; Thompson *et al.*, 2009). There is, however, no clear distinction between the two cell types as cells of either phenotype have been found throughout the graft, and therefore rather represent the majority of cells (Thompson *et al.*, 2005; Thompson *et al.*, 2009) (ii) only cells of the A9 phenotype were able to normalise cFos expression in the striatum after 6-OHDA lesions and were able to completely restore the behavioural deficits induced by the lesion twelve weeks post transplantation as assessed via amphetamine-induced rotation and the cylinder test. In contrast, grafts that were composed of A10 type cells did not improve behavioural deficits (Grealish *et al.*, 2010a).

## 1.12 6-Hydroxydopamine

Animal models that mimic all or certain aspects of the human disease are essential in understanding disease mechanisms and possible treatment options. The most commonly used agent to induce a unilateral rat model of PD is 6-OHDA (Ungerstedt, 1968; 1971; Beal, 2001; Dawson *et al.*, 2002). When injected directly into the brain 6-OHDA is neurotoxic and selectively kills catecholaminergic neurons (DA, adrenaline, and noradrenalin). 6-OHDA does not cross the BBB and therefore must directly be injected into the brain via stereotaxic surgery (Bove *et al.*, 2005; Rang *et al.*, 2007). It is theorised that 6-OHDA is brought into the cell via the DAT and then oxidation leads to the production of reactive oxygen species and para-quinone (Dauer & Przedborski, 2003; Bove *et al.*, 2005).

It was discovered that direct lesions to the SN did not only lead to degeneration of DAergic neurons in this structure but furthermore a depletion of tyrosine-hydroxylase (TH) in the neuron terminals within the striatum (Ungerstedt, 1968). The depletion in the striatal region occurred in a time-delayed manner relative to the injection to the SN. Furthermore it was discovered that unilateral lesioned animals displayed lateralised motor impairments (Ungerstedt, 1968; Ungerstedt & Arbuthnott, 1970; Ungerstedt *et al.*, 1974; Ljungberg & Ungerstedt, 1976). 6-OHDA lesioned rats that were injected with a DA agonist displayed a rotational behaviour (Ungerstedt &

Arbuthnott, 1970; Ljungberg & Ungerstedt, 1978; Ungerstedt *et al.*, 1978). Depending on the percentage of neuronal loss, animals that are challenged with the indirectly-acting DA agonist methamphetamine (stimulates DA realase and blocks reuptake), circle towards the side of the lesion. In near-complete unilateral lesions there is an imbalance in DA innervations between the intact and the lesioned striatum. Methamphetamine acts only on the un-lesioned side causing the animal to rotate towards the lesion, as the release of the neurotransmitter is restricted to the intact side only. Conversely, animals that are injected with the true agonist apomorphine at (very) low doses rotate away from the side of the lesion, as the DA-receptors up-regulate after dopamine depletion and therefore the lesioned side is rendered supersensitive to (post-synaptic) DA activation (Ungerstedt & Arbuthnott, 1970; Zigmond & Stricker, 1972; Ungerstedt *et al.*, 1975; Kelly & Moore, 1976; 1977; Truong *et al.*, 2006; Torres & Dunnett, 2007; Grealish *et al.*, 2008). Lesions to either the ascending axonal bundle of the ventral mesencephalic DAergic neurons or to the neuronal terminals within the striatum produce contralateral motor impairments (Kirik *et al.*, 1998; Grealish *et al.*, 2008). Whereas lesions to the medial forebrain bundle lead to near complete depletion of TH-positive cells, terminal lesions, when aimed at the dorsal striatum, do not lead to extensive depletion of TH-ir neurons in the VTA (Zigmond & Stricker, 1972; Truong *et al.*, 2006; Grealish *et al.*, 2008). As a model of PD, 6-OHDA is usually injected unilaterally into SN, the medial forebrain bundle or into the striatum. The unilateral model has been used extensively and has been shown to produce deficits on the side of the body contralateral to the hemisphere in which the injection was administered in various motor tests, such as the cylinder test, adjusting steps test, drug induced rotations, corridor test and operant tests of behaviour (see section below) (Ungerstedt & Arbuthnott, 1970; Ungerstedt, 1971; Zigmond & Stricker, 1972; Schallert *et al.*, 1978; Carli *et al.*, 1985; Carli *et al.*, 1989; Montoya *et al.*, 1990; Schallert *et al.*, 2000; Dowd & Dunnett, 2004; Dowd *et al.*, 2005a; Iancu *et al.*, 2005). Bilateral injections are less common as, dependent on the extent of DAergic depletion, lesioned animals usually drop in weight and/or have an inability to feed themselves (Zigmond & Stricker, 1972; Dunnett *et al.*, 1983b; Bove *et al.*, 2005). Due to this, extensive post-operative care with tube feeding and daily injections with glucose-saline to stabilize the animals' weight might be necessary.

The major drawbacks of the 6-OHDA lesion model of PD are that the lesion only produces a subset of the impairments seen in PD. However, none of the other animal models produce the characteristic tremor either, at least in rats. Furthermore, as with all toxin based models, the onset of the "disease" is acute and not progressive as

seen in the human condition and the 6-OHDA lesion model does not produce Lewy-bodies that are found in the brains of human PD patients. Therefore, it is important to keep in mind that it is a model of DA depletion, the major hallmark of PD, and not a model of PD itself.

## 1.13 Modelling PD though α-synuclein overexpression

Linking α-synuclein to genetic causes of PD pathology has sparked research into modelling the overexpression of the gene in animals as a research tool (Kirik *et al.*, 2008). The first three genetic mutations of the protein α-synuclein that were discovered in familial PD were A53T (Polymeropoulos *et al.*, 1997), A30P (Kruger *et al.*, 1998), and E46K (Zarranz *et al.*, 2004).

Overexpression of these human forms of α-synuclein via viral vectors has been shown to be toxic to SN DA cells and have several advantages above transgenic mice in that (i) the transgene can be introduced at any time point in the animal's life, (ii) higher transgene expression is achieved, (iii) slower progression of the disease as compared to the acute insult by catecholaminergic neurotoxins (iv) the overexpression can be induced in other models than mice, (v) vector injections can be aimed at specific brain structures via stereotaxic surgery, (vi) unilateral disease models can be created so that each animal can serve at its own within-subject control, (vii) bilateral models can be created which more closely resemble the human condition and are required for testing of higher cognitive functions and (viii) due to manipulations of the vector more than one gene can be mutated, i.e. generating models of A53P or A30P (Kirik *et al.*, 2002; Kirik *et al.*, 2003; Kirik & Bjorklund, 2003; Maingay *et al.*, 2005).

Although transgenic α-synuclein mouse models exist, they only show a limited reduction in tyrosine-hydroxylase immunoreactive (TH-ir) density in the striatum and no detectable cell loss of TH-ir cells in the SN (Blandini & Armentero, 2012). We therefore focus here on the overexpression of α-synuclein via viral vectors. For a review on transgenic mouse models of PD please see (Fleming *et al.*, 2005; Harvey *et al.*, 2008).

After vector injection into the SN the first α-synuclein-ir deposits can be found as early as 3 weeks post injection and 10 weeks after injections PD like pathology in the form of α-synuclein-ir inclusions as well as swollen axons and dendrites have been

reported (Kirik & Bjorklund, 2003; Chu *et al.*, 2012). Cell bodies in the SN appear shrunken with α-synuclein-ir deposits, which resemble the Lewy bodies found in the brains of PD patients (Kirik *et al.*, 2002; Kirik *et al.*, 2003; Kirik & Bjorklund, 2003). The α-synuclein vector model has been used successfully in rodents and non-human primates, in that it can induce nigral cell loss, striatal DA depletion and the formation of inclusions (Lo Bianco *et al.*, 2002; Eslamboli *et al.*, 2003; Kirik *et al.*, 2003; Kirik & Bjorklund, 2003; Eslamboli *et al.*, 2007; Koprich *et al.*, 2011). The loss of TH-ir cells in the SN is highly variable and depletion ranges between 30% and 80% have been reported (Kirik *et al.*, 2002; Klein *et al.*, 2002; Lo Bianco *et al.*, 2002; Lo Bianco *et al.*, 2004; Chung *et al.*, 2009; Koprich *et al.*, 2010; Koprich *et al.*, 2011; Chu *et al.*, 2012; Decressac *et al.*, 2012a; Decressac *et al.*, 2012b; Lundblad *et al.*, 2012).

Despite a lack of long term behavioural studies (>6 month), the tests that were conducted show that the cell loss and striatal TH depletion occurs over a time frame of 3 to 16 weeks, after which expression of α-synuclein, striatal TH depletion and TH-ir SN cell degeneration is maximum. Although α-synuclein can still be detected in cells up to 6 month post injection, clear signs of recovery were found in the form of missing α-synuclein-ir inclusions, and recovery of striatal density (from 50% to 80%) (Kirik *et al.*, 2002; Koprich *et al.*, 2010; Koprich *et al.*, 2011). However, this recovery was not complete, i.e. striatal DA content was still at 50% of control and the deficits could be partially reinstated by alpha-methyl-para-tyrosine (Kirik *et al.*, 2002), suggesting that remaining cells compensate for the loss by sprouting, more DA release and up-regulation of synaptic transport. Long term assessment for 12 months after overexpression of the A30P variant in the SN revealed a 53% loss of SN DA neurons and α-synuclein-ir deposits in the SN and striatum but failed to show any behavioural deficits (Klein *et al.*, 2002). The assessment method in the reported study was unusual as they activated rats on a low dose (2mg/kg) d-Amphetamine injected intra-muscularly, instead of the usual method using 2.5mg/kg methamphetamine or 2.5-5.0mg/kg d-amphetamine injected i.p., therefore the threshold of depletion for assessment of motor deficits may not have been reached. This posed problems for the use of this model for behavioural testing as (i) a depletion of striatal TH of >50%-70% is needed to show robust behavioural deficits and (ii) the lesion should be stable and not be prone to spontaneous behavioural recovery as any effects by intervention cannot be assigned to the intervention with confidence (Kirik *et al.*, 1998; Kirik *et al.*, 2002; Dowd & Dunnett, 2004; 2005b).

A first attempt to increase the transfection rate by using a woodchuck hepatitis virus posttranscriptional regulatory element did increase expression by 4-5 fold and led to degeneration of TH-ir cells in the SN by 70% of control (Decressac *et al.*, 2012a; Decressac *et al.*, 2012b). The degeneration reported was progressive and occurred over a period of 8-16 weeks with the first behavioural effects being detectable after 3 weeks post transfection (Klein *et al.*, 2002). Animals displayed a bias on both, the cylinder test and amphetamine-induced rotations (Decressac *et al.*, 2012a). However, unfortunately the motor deficits were small compared to neurotoxic lesions (Kirik *et al.*, 2002; Decressac *et al.*, 2012a) and although analysis was carried out up to 16 weeks post injection it is still possible that the animals would undergo the same recovery as the ones in the Kirik et al. (2002) study. Interestingly the accumulated data suggests that the levels of α-synuclein between cells might vary and that some cells, although expressing the protein, will recover because the critical threshold for neurodegeneration to occur has not been reached (Kirik *et al.*, 2002). Furthermore some cells do survive for 5 months post injection whilst expressing the mutant α-synuclein (Lo Bianco *et al.*, 2002). Other problems with the use of the α-synuclein model for PD, is that the area affected as well as the lesion extent is difficult to define. Whereas 6-OHDA can be used to selectively lesion the dorsal striatum and cause reliable deficits in motor and cognitive functions, the viral vector model has not been shown to produce well defined depletion in one area only (Kirik *et al.*, 1998; Koprich *et al.*, 2010; Koprich *et al.*, 2011; Decressac *et al.*, 2012a). For the purpose of brain repair a lesion in the dorsal striatum is necessary to obtain stable deficits, which can then be used for testing therapies such as DBS or cell replacement. The α-synuclein model, at the present time, does not allow to pursuit those strategies as (i) the lesion is not well defined, (ii) the deficit induced is prone to spontaneous recovery, (iii.) the behavioural deficits are small (i.e. rotations: 3rpm compared to >6rpm after 6-OHDA insult), (iv) the 'success rate' is relatively small as on average 25% of animals display a depletion of sufficient size to display motor problems whereas in the 6-OHDA lesion model a near complete depletion can be achieved relatively easily (>90%) (Torres *et al.*, 2011), and (v) adequate control groups are lacking for comparison as protein overload can be toxic to cells whereas empty vectors are not sufficient (Kirik *et al.*, 2002; Klein *et al.*, 2006; Decressac *et al.*, 2012a; Decressac *et al.*, 2012b).

Although the α-synuclein model is a beneficial addition to the scientific toolbox available and will be especially valuable in investigating neuroprotective agents, at present the degree of depletion is not high enough to produce reliable and robust

behavioural deficits which are needed for the assessment of cell replacement therapy (i.e. only 25% of the animals injected with the virus display behavioural deficits). At the moment the α-synuclein model is rather useful for investigations into the underlying neuropathology of PD until a higher level of DA depletion is achieved.

## 1.14 MPTP

In California, in 1982 four patients were discovered who developed parkinsonism within a week after use of a synthetic heroin substitute. The patients were reported to have developed all of the hallmark symptoms of PD such as immobility, postural instability, impairments in speech, eye movement and blinking, as well as shuffling gait and bradykinesia (Langston *et al.*, 1983). This was striking as usually juvenile cases are rare and the progression of the disease is typically slow, taking years. All patients were responsive to l-DOPA treatment and symptoms returned completely upon withdrawal of the medication. One of the patients died within two years after a drug-overdose and examination of his brain revealed a loss of pigmented cells in the SN (caudal region most affected). Although, in contrast to idiopathic PD patients, other nuclei, such as the locus coeruleus and dorsal motor vagus nucleus, were not affected, the behavioural symptoms, the responsiveness to l-DOPA and the loss of cells in the SN led to the conclusion that the synthetic drug intoxication can lead to a form of PD (Langston *et al.*, 1983). The compound injected was subsequently identified to consist of 1-methyl-4-phenyl-1,2,5,6-tetrahyropyridine (MPTP) with traces of 1-methyl-4-phenyl-4-propionoxy-piperidine (MPPP) (Langston *et al.*, 1983). After injection, MPTP readily crosses the BBB and is taken up by glial cells. In those glial cells MPTP is converted via Monoamine Oxidase-B (MAO-B) to $MPDP^+$ and then to $MPP^+$, which is the toxic agent. Although it is unknown how $MPP^+$ leaves the glial cell, it is taken up by the DA neurons via the DAT (Schapira, 1999a; b). Inside the DA neuron it can be clustered inside synaptic vesicles via the VMAT-2, where it can interact with cytosolic enzymes and build up in the mitochondria (Dauer & Przedborski, 2003). Inside the mitochondria it can inhibit enzyme complex I of the electron transport chain and subsequently lead to a reduction in ATP levels and an increase in reactive oxygen species (Schapira, 1999a; b). The relative selective neurotoxicity to DAergic cells of the SN does make MPTP an excellent research tool to model DA loss in animal models of PD. Since its discovery it has been applied to model PD in mice, rats, and non-human primates (Lange, 1989; 1990; Sundstrom *et al.*, 1990; Sedelis *et al.*, 2000a; b; Jenner, 2003; 2009). Although the MPTP monkey has been valuable in modelling the disease, the application in mice has shown that

toxicity of the compound varies with age, gender, and strain (Ricaurte *et al.*, 1987; Sundstrom *et al.*, 1990; Irwin *et al.*, 1992; Sedelis *et al.*, 2000a; b; Hofele *et al.*, 2001). Younger mice are relatively resistant to MPTP lesions and display a high degree of behavioural recovery whereas in older animals the compound is often lethal (Langston *et al.*, 1983; Langston *et al.*, 1987; Irwin *et al.*, 1992; Sedelis *et al.*, 2000a). MPTP has also shown higher toxicity in male mice compared to females (Ookubo *et al.*, 2009), and certain strains are more resistant to MPTP induced DA cell degeneration than others (Sedelis *et al.*, 2000a; b; Hofele *et al.*, 2001). Most of the effects of toxin resistance have been attributed to reduced levels of MAO-B, which is responsible for converting MPTP into the toxic agent $MPP^+$. Furthermore, systemic injection in the rat does not lead to SN DA cell degeneration and stereotaxic injection is required. Other concerns with the use of MPTP as a research model are the mode of administration as the standard acute lesions are achieved via systemic injections and therefore lead to a bilateral depletion model. As the aim of the present work is to assess unilateral deficits and using the intact hemisphere as within-subject control, the unilateral stereotaxic injection is more appropriate. Furthermore, although MPTP can be handled if the right precautions are met, it still is highly toxic to man, and suitable precautionary measures must be taken.

## 1.15 Other models

Other toxins that are used to model PD in animals were discovered, after structural similarities were seen between MPTP and certain herbicides/pesticides. The two most common are rotenone and paraquat (Blandini & Armentero, 2012). The focus here is these and the reader is referred to the review by Caudle et al. (2012) for an overview about industrial toxins (Caudle *et al.*, 2012).

Chronic injection into the rat of rotenone leads to relative selective degeneration of DAergic SN cells and to the formation of α-synuclein positive inclusions (Blandini & Armentero, 2012). The disadvantages with the rotenone model are mainly its high variability and the high mortality as the compound also attacks cells in the liver, gut, heart and kidneys (Blandini & Armentero, 2012). Recent advances have been made by characterisation of direct injection of rotenone into the brain of laboratory rats. Injection at any level of the nigro-striatal pathway resulted in loss of TH from the striatum and a loss of TH-ir DA cells in the SN (Saravanan *et al.*, 2005; Sindhu *et al.*, 2005; Mulcahy *et al.*, 2011) without the adverse effects of increased weight loss and increased levels of mortality. Recently a combination of the AAV-α-synuclein model

and subsequent (13 weeks) intra-nigral infusion of rotenone has shown to increase the depletion of striatal DA depletion, SN TH-ir cell loss and contralateral motor deficits (Mulcahy *et al.*, 2012). Despite this, the specific neurotoxicity has been questioned and lesions beyond the boundaries of the injection site have been reported (Klein *et al.*, 2011). Paraquat does selectively destroy SN cells but its toxic effect is relatively weak and the resulting cell loss in range of 20-30% (Blandini & Armentero, 2012). The two most common pharmacological models are reserpine and Haloperidol. Although they do mimic some aspects of the disease they are limited in that they are transient, and do not show any pathology. Reserpine injection results in a temporary loss of striatal and nigral DA (monoamines) for 2-3 days via inhibition of the VMAT-2. Reserpine injected rats do display some PD like features, such as rigidity and akinesia, which could be reversed via l-DOPA administration (Carlsson *et al.*, 1957; Carlsson *et al.*, 1968; Duty & Jenner, 2011). Haloperidol on the other hand does not mimic any of the parkinsonian features seen in patients, but nevertheless, injection of haloperidol has an antagonistic effect on D2 and D1 receptors on striatal MSNs (Duty & Jenner, 2011).

## 1.16 Rationale for using 6-hydroxydopamine

Although all of the above mentioned models have major advantages and drawbacks in their use as tools to model PD, here I chose to use the 6-OHDA lesion model in the following experiments for a combination of reasons. Firstly, practically working with 6-OHDA is much less dangerous as it only affects humans when administered into the brain whereas the other toxins can cross the BBB, e.g. MPTP has induced Parkinsonism in a chemist working with the substance (Langston & Ballard, 1983). Furthermore, this model has been widely used for over 40 years of research and therefore results obtained are more easily comparable with the existing literature. Various studies have assessed the effects of DAergic depletion on animals' behaviour and the effect of neuroprotective agents, DA replacement and neuronal grafting in this lesion model. Thirdly, the aim of this work is to investigate the behavioural effects of DAergic depletion therefore the lack of protein inclusions is acknowledged but regarded as a minor drawback concerning this research. Additionally, the 6-OHDA model allows, via selective lesions, depletion of DA dissociation of nigro-striatal degeneration from other aspects of PD. As the main aim of the present thesis is the characterisation of the lesion induced deficit for subsequent transplantation, a large DA depletion model is thought to be more relevant to the clinical case where patients with a more 'pure' parkinsonism, that is

highly responsive to l-DOPA therapy, display greater therapeutic benefits by the cell replacement.

Importantly, Kirik et al. (1998) showed that 6-OHDA can be used to produce lesions of various extents, depending on the site of injection and the concentration used. As the main goal is to develop and characterize models that are sensitive measurements of graft induced recovery a stable lesion that does not show signs of spontaneous recovery is warranted. Since DA depletion generally has to be greater than 60%-70% before the first behavioural abnormalities can be detected, we aimed for lesions that lead to a high depletion (Kirik *et al.*, 1998; Truong *et al.*, 2006).

Therefore, although 6-OHDA does not mimick the pathogenic process as done by MPTP or α-synuclein, which would be important in studying neuroprotection, it does provide a reliable lesion model that is reproducible and stable over time, which are important factions for studying DAergic cell replacement approaches and functional recovery.

## 1.17 Operant analysis of behaviour

Operant analysis of animals' behaviour has several advantages to simple tests of motor and sensory-motor tasks. Although training animals to perform on operant tasks at a high level can be time consuming, many animals can be trained at the same time in an objective manner, largely eliminating the effects of experimenter bias. Whereas simple motor tasks are dependent on the expertise of the experimenter, in operant tasks the animals are placed in a computer controlled environment and tested independently from the experimenter. Furthermore high numbers of trials can be conducted leading to more sensitive testing. The large number of trials gathered within a 30 minute session facilitates tight variance and power/sensitivity to detect small effects. This does not only make behavioural testing more efficient, but it also reduces the number of animals required to detect medium or small sized effects, which provides an additional advantages in the 3Rs (Replacement, Refinement, Reduction) and animal welfare. A large number of parameters, including reaction and movement time latencies accurate to the millisecond, can be recorded at the same time, allowing for a detailed functional analysis of the determinants of behavioural responses, rather than simple empirical assays of behavioural deficits.

In operant analysis of behaviour the animal can be trained to learn arbitrary 'stimulus-response' or 'action-outcome' associations. Often a stimulus light signals that a certain action is required by the animal in order to obtain a reward in the form of an incentive reward, such as sucrose pellets. Lesions to sub-regions of the lateral and medial striatum have shown that the striatum is heterogeneous in function. Whereas lesions to the dorsal/lateral part prevent the formation of habits, lesions to the medial part of the striatum disrupt action-outcome related behaviours (Yin & Knowlton, 2002; 2004; Yin *et al.*, 2004; 2005a; Yin *et al.*, 2005b; Yin & Knowlton, 2006; Yin *et al.*, 2006). During initial task performance animals do not respond out of habit to the stimulus light that is presented but they rather focus on the reward to be obtained. This type of behaviour is goal-directed and it has been shown that devaluation of the reward does influence the behaviour of the animal (Yin & Knowlton, 2002). Overtrained animals start to develop a habit, which is association of the stimulus when the association with the outcome is lost, i.e. not influenced by outcome devaluation.

All of the above mentioned studies utilise cell body lesions in the striatum by means of infusion of an excitotoxic as quinolinic or ibotenic acid, and/or temporarily inactivation, to produce stable and defined lesions. DA depletion via 6-OHDA infusion has been shown to produce variable lesions, which can undergo compensation and spontaneous behavioural recovery can occur. Remaining cells up-regulate their DA release and sprouting of terminals has been reported (Acheson & Zigmond, 1981; Robinson & Whishaw, 1988). Furthermore, striatal cells that are depleted of their DAergic input will up-regulate their DA receptors. Remaining tonic striatal DA by neighbouring innervations make it difficult to produce defined lesions as can be done by aforementioned neurotoxins. Although the literature on habit formation using DA lesions is sparse, the accumulated evidence suggests (lateral) DA depleted rats will continue displaying goal-directed behaviour when control animals are habitual (Faure *et al.*, 2005; Faure *et al.*, 2010).

The first studies that investigated unilateral striatal DA depletion by injecting the selective neurotoxin 6-OHDA into the striatum of laboratory rats on operant tasks showed that animals developed a deficit when responses had to be made to the side of the body contralateral to the lesioned hemisphere (Carli *et al.*, 1985; Carli *et al.*, 1989). Animals that were trained in the 9-hole box apparatus on a lateralised choice reaction time task had to perform a sustained nose-poke into an illuminated centre hole. After a delay, a stimulus light to either the left or the right side of the animals'

head indicated the response hole to which a nose poke would result in the delivery of a food reward. Animals that were lesioned showed a deficit in their accuracy when a response was required contralateral to the lesion (Carli *et al.*, 1985; Carli *et al.*, 1989). Using a slightly different version of this task, in which lower stimulus-response associability is achieved by requiring rats to respond to the hole on the opposite side of the light stimulus, it was revealed that the observed motor deficit was not sensory in nature. Here, animals continue to demonstrate a motor deficit on the side of the body contralateral to the side of the lesion, similar to animals that have to respond to the same side where the stimulus is presented, while demonstrating an intact ability to perceive the visual stimulus on the contralateral side (Carli *et al.*, 1985; Carli *et al.*, 1989; Dowd & Dunnett, 2005a; b). Brown and Robbins (1989) modified the paradigm of Carli et al. (1985) and instead of using a bilateral hole configuration they trained the animals to report the occurrence of a stimulus light to either a near or a far hole on the same side of the animal's head. After training, they subsequently lesioned the right striatum in one group of animals and the left striatum in the other cohort, via intracerebral injection of 6-OHDA. Animals that were lesioned ipsilateral to the side of response did not show a deficit on the operant task whereas animals that were lesioned on the side contralateral to the response location showed an interesting deficit. Contralateral lesioned animals were still able to register the occurrence of the stimulus and execute a response but they were biasing their responses almost completely too the nearer of the two response locations. Interestingly, when given a bilateral choice, lesioned animals developed a bias towards the side ipsilateral to the lesion, but when given no choice they respond to the relative ipsilateral location (Brown & Robbins, 1989a). In subsequent experiments it has been shown that near complete unilateral DA depletion from the striatum via injection of the neurotoxin into the ascending DAergic bundle can produce a stable deficit whereas terminal lesions can show spontaneous recovery over the weeks of post lesion testing (Carli *et al.*, 1985; Dowd & Dunnett, 2005a; b). Therefore, only severe DA depletion leads to a stable lesion model that is suitable for long term behavioural analysis and subsequent evaluation of cell replacement strategies. Although spontaneous recovery occurs in partially lesioned animals, the deficit can usually be re-instated by further DAergic depletion by injection of α-methyl-p-tyrosine, an inhibitor of DA synthesis (Carli *et al.*, 1989; Dowd & Dunnett, 2004). Other studies have shown reaction time impairments after unilateral lesions that are suspected to be the result of a movement initiation deficit, similar to that seen in humans with PD (Amalric & Koob, 1987; Jahanshahi *et al.*, 1993; Blokland, 1998). The measurement of reaction time deficits mainly depends on the sensitivity of the apparatus and nature of the

required response. Reaction time impairments have been shown in the 9-hole box but not in the Skinner box in striatal lesioned animals that received quinolinic acid lesions (Brasted *et al.*, 1998). In order to test the efficacy of treatment options for PD such as neural restoration or cell replacement it is important that the tests that are employed are sensitive enough to detect changes in the animals' behaviour. Whereas subtle improvement may be missed when the crude and simple tests of motor asymmetry are applied, operant analysis of behaviour can detect more subtle changes (Dowd & Dunnett, 2004; 2005b).

## 1.18 Reaction time testing in rodents

The time to react to external stimuli can be assessed using one of three possible paradigms, (i.) simple reaction times, (ii.) choice reaction times, and (iii.) cued reaction times (Hauber, 1998). Whereas during voluntary movement the stimulus to execute the movement is internal, in simple and choice reaction time tasks there is an external imperative stimulus that signals the animal to initiate the movement. From this external stimulus the animal then has to select the appropriate response profile (motor programs) and subsequently initiate the movement. Importantly, in simple reaction time tasks all the information about 'where' the to-be-executed response has to be directed is available to the animal at all times. The only parameter of uncertainty is 'when' the response has to be executed. This advanced information allows selecting and assembling the movement plan already before the imperative signal is perceived. In contrast, in choice reaction times there is a high level of uncertainty of 'where' and 'when' the response has to be made. Information is only available with the onset of the stimulus and therefore motor preparation cannot start before this information is available to the animal (Hauber, 1998). Exeptions to this rule are cued choice reaction time tasks. In general, a pre-cue is given to the animal with information regarding where the response is located in space, therefore some of the information can be given to the animal in advance. Usually, the cue is given at different times before or together with the imperative stimulus, thereby assessing the ability of the animal to make use of this advanced information.

Reaction time testing in rats has a long history in analysing the deficits induced by unilateral DA depletion and allows for the dissociation of multiple task parameters as the time to initiate an execute (lateralised) responses. Whereas reaction time testing in humans and non-human primates is often accompanied by recording electromyographic activity, this is generally neglected in the rat (Hauber, 1998).

Therefore it is difficult to further separate the time of stimulus detection, preparing of a motor plan and the signal sending to the muscle. A great deal of research has shed light on the lesion-induced deficit and especially on the role of the DAergic system therein. Early studies have investigated whether the deficit in reaction time responding is more likely to resemble bradykinesia (slowness of movement execution) and/or akinesia (initiation of movement), both symptoms are known to be caused by dysfunction of the basal ganglia, or if they are caused by deficits in sensory perception (Hauber, 1998). Using a conditioned-turning test in rats, Dunnett and Bjorklund (1983) have shown that unilateral 6-OHDA lesions do disrupt the acquisition and the maintenance of the conditioned response when they had to turn contralateral to the lesion, but not ipsilateral. The authors concluded that the deficit caused by the lesion was a disruption of movement initiation rather than movement execution, per se (Dunnett & Bjorklund, 1983). Furthermore, when animals are required to make a sustained hold before the imerative stimulus signals the execution of the (lateralised) response, longer 'fore-periods' led to shorter reaction times than shorter 'fore-periods' (Brown *et al.*, 1991; Courtiere *et al.*, 2011). Another observation was that animals do respond faster when a congruent cue is given rather than an incongruent (Courtiere *et al.*, 2011). Partial 6-OHDA lesions into the dorsal striatum do not impair the congruency-effect but does impair the effect of fore-period, although during the first post-operative week of testing (Courtiere *et al.*, 2011). Using the choice reaction time task Carli and colleagues elegantly showed that lesioned rats indeed displayed deficits in movement initiation, rather than a deficit due to primary sensory perception and/or primary movement execution (Carli *et al.*, 1985). When comparing two groups of rats that, after a responding to a centre hole, had to report the occurrence of a lateralised stimulus light either with a nose poke into, or away, from the location of the stimulus, they found that both groups of rats could accurately detect the stimulus, therefore excluding a deficit in sensory perception (Carli *et al.*, 1985). In a further study they demonstrated that the deficits was caused by depletion in the striatum whilst additional depletion of DA from the nucleus accumbens did not have additive effects (Carli *et al.*, 1989). On a simple reaction time task bilateral terminal 6-OHDA lesions impaired reaction time performance whereas bilateral lesions to the nucleus accumbens did not have a significant effect (Amalric & Koob, 1987). This is in accordance with the view that the nucleus accumbens is rather involved in reward signalling and motivational aspects of an operant task than reaction time performance, per se (Cousins *et al.*, 1993; Salamone *et al.*, 1994). Both operant studies showed that next to the accuracy deficit, lesioned rats displayed an increase in reaction time latencies (Carli *et al.*, 1985; Carli *et al.*,

1989). Furthermore, rats that are trained to release a lever upon an imperative signal and receive the DA antagonist (receptor blocker) haloperidol into the striatum display an increase in reaction time latencies, a reduction in the number of usable trials, and an increase in anticipatory trials (Amalric & Koob, 1989) to a similar extent to bilateral 6-OHDA lesions (Amalric & Koob, 1987). Interestingly, the increase in reaction time latencies, which was accompanied by an increase in the number of delayed responses in a simple lever release task, could be reduced by subsequent bilateral lesions of the STN (Baunez *et al.*, 1995b). Similar, intra-striatal administration of d-amphetamine led to a speeding-up of reaction time latencies (Baunez *et al.*, 1995a).

In an interesting study by Brown and Robbins (1991), rats were tested in choice and cued conditions within the same session; the brightness of the bilateral presented stimuli indicated an ipsilateral or contralateral response and rats were presented either simultaneously with an auditory signal as imperative signal or the imperative auditory cue was delayed after the presentation of the visual stimuli, thereby, according to the authors, turning the task into a simple reaction time task (Brown & Robbins, 1991). Unilateral DA depleted rats were still able to make use of the advanced information as shown by superior performance in the simple reaction time setting compared to the choice setting. Although an elegantly designed study, the effects of pre-cueing are addressed as the authors state themselves that '*[…], fully cued reaction time tasks are equivalent to simple reaction time tasks on neither theoretical nor empirical grounds*' (p.866; Gauntett-Gilbert & Brown, 1989). There is still a level of uncertainty until the cue is presented and it is not clear that lesioned animal's have enough time to incorporate the information to prepare the motor-plan (Gauntlett-Gilbert & Brown, 1998). Nevertheless, simple reaction time was faster than choice reaction time, an effect that was not disturbed by the 6-OHDA lesion, and it was therefore concluded that DA depleted rats are capable of integrating advanced information (Brown & Robbins, 1991). Systemic administration of amphetamine furthermore enhances motor readiness on a simple reaction time task (Brown *et al.*, 1996).

Taken together, the above studies demonstrate the effect of striatal DA loss on reaction time, whereas loss in the nucleus accumbens was not effective in inducing similar deficits. Dopamine depletion in the striatum leads to an impairment in movement initiation, whereas primary sensory and/or motor deficits can be excluded (Carli *et al.*, 1985). The reintroduction of DA to the depleted striatum via direct infusion of the neurotransmitter (Baunez *et al.*, 1995a) or by means of engrafting DA-

rich fetal cells (Lelos *et al.*, 2012) did reduce reaction time after the lesion. Importantly, the right amount of DA is critical, as too much or little can have negative or no effects, respectively. The engraftment of cells that can regulate the DA release will therefore be more likely to show therapeutic benefits, if appropriate re-inneravation is achieved, than infusion via, i.e. a minipump, into the brain. Choice reaction time testing is able to dissociate impairments in stimulus perception, movement initiation and movement execution.

## 1.19 Simple tests of motor asymmetry

The 6-OHDA unilateral lesion model has been a valuable research tool for almost 4 decades since its introduction. Rats that received unilateral lesions along the nigro-striatal pathway have been shown to develop contralateral motor impairments and show a form of contralateral sensori-motor neglect. Simple tests of DA imbalance, such as the drug-induced rotation test, or tests of motor function such as the cylinder test, the adjusting steps test, the staircase test, single pellet reaching test, and the corridor test have been shown to be sensitive to unilateral DA depletion (Ungerstedt & Arbuthnott, 1970; Schallert *et al.*, 1978; Montoya *et al.*, 1990; Montoya *et al.*, 1991; Olsson *et al.*, 1995; Kirik *et al.*, 1998; Nikkhah *et al.*, 1998; Schallert *et al.*, 2000; Dowd *et al.*, 2005a; Marin *et al.*, 2006; Torres & Dunnett, 2007; Grealish *et al.*, 2008)

Although all of these tests have been valuable both in investigating the function of DA in producing motor behaviour and in the ability of DA rich transplants to restore the lesion-induced deficits, they have some limitations.

## 1.20 The 6-OHDA mouse lesion model of PD

The 6-OHDA lesion model has been the gold standard as a PD model for researchers with the vast majority of studies conducted using the rat as the model organism. Despite 6-OHDA mouse lesion models being used over the years there is little standardisation about the parameters used. Across the literature (see Table 1.1) there is a huge variation in background strain, injection volumes, toxin concentration, and even lesion coordinates that target the same structure (Smith & Heuer, 2011). Furthermore, there has been little systematic characterisation in the mouse with regards to behavioural assessment and their correlations with lesion extent. In the rat, several studies have established thresholds and indicators that allow researchers to identify if an animal is well lesioned or not (Kirik *et al.*, 1998; Torres & Dunnett, 2007; Grealish *et al.*, 2008).

With the advent of genetic mouse models and the relative ease with which these can be generated compared to the rat there is a need for a thorough characterisation of the mouse lesion model. The rat bundle lesion model provides the most reliable lesion with a near complete depletion of TH-ir DA cells on the side of injection. A direct transfer to the mouse has been proven difficult for several reasons: (i) the lesion success rate has been more variable than in the rat, (ii) bundle lesions in particular are associated with a very high mortality rate, although the same procedure in rats generally does not cause mortality; (iii) parameters for the lesion procedure have not been systematically investigated, and (iv) there are not standardised behavioural tests characterised that can distinguish lesioned from partially lesioned mice.

All these factors are very important for studies that require a full lesion, i.e. for the development of dyskinesias, and for the investigation of long term treatment effects, e.g. cell replacement therapy. Partially lesioned animals can display spontaneous recovery and the effects of any therapeutic intervention can therefore be masked.

| Concentration | Dose | Volume | Target | Coordinates | Strain | Sex | Reference |
|---|---|---|---|---|---|---|---|
| **4 µg/µl** | 16 µg | 4 µl | Striatum | AP= +5 (to occipital structure)<br>ML= -2.2<br>DV= -3.5 | Swiss-Webster | M | (Torello *et al.*, 1983) |
| **4 µg/µl** | 16µg | 4 µl | Striatum | AP= +0.5<br>ML= -2.4<br>DV= -3.1 | C57Bl/6 | M/F | (Randall, 1984) |
| **3.32 µg/µl** | 13.3 µg | 4 µl | Striatum | AP= +4.8 (from Lambda)<br>ML= -1.7<br>DV= -3.0 and -2.7 | MNRI | M | (Brundin et al., 1986) |
| **3 µg/µl** | 3.9 µg or 5.4 µg | 1.3 µl or 1.8 µl | Striatum | AP=-1.2<br>ML=±1.1<br>DV=-5.0 | CBA | F | (Iancu et al., 2005) |
| **1.6 µg/µl** | 2.4 µg | 1.5 µl | SN | AP=-3.0<br>ML=-1.2<br>DV=-4.5 | NMRI | F | (Grealish et al., 2010b) |
| **4 µg/µl** | 16 µg | 4 µl | Striatum | AP= +0.5<br>ML= -2.4<br>DV= -3.1 | C57Bl/6 | M | (Mandel & Randall, 1990 |
| **2 µg/µl** | 4 µg | 2 µl | Striatum | AP= +0.4<br>ML= -1.8<br>DV= -3.5 | C57/Bl6 | M | (Bensadoun et al., 2000) |
| **3 µg/µl** | 3 µg | 1 µl | MFB | AP= -1.2<br>ML= -1.2<br>DV= -4.75 | C57Bl/6 | M | (Lundblad et al., 2004) |
| **3 µg/µl** | 6 µg | 2x2 µl | Striatum | $AP_1$ = +1.0 and +0.3<br>ML= -2.1 and -2.3<br>DV = -2.9 and -2.9 | C57Bl/6 | M | (Lundblad et al., 2004; Lundbl al., 2005) |
| **2.5 µg/µl** | 10 µg | 2x 2 µl | Striatum | AP= +0.5<br>ML= +2.4<br>DV -4.0 and -3.0 | C57BL/6J/ OlaHsd | M | (Pavon et al., 2006) |
| **3 µg/µl** | 12 µg | 2x 2 µl | Striatum | $AP_1$ = +1.0 and 0.3<br>ML= - 2.1 and -2.3<br>DV= -3.2 and -3.2 | C57Bl/7 | M | (Santini et al., 2007) |
| **4 µg/µl** | 8 µg | 2 µl | Striatum | AP= + 0.4<br>ML= - 1.8<br>DV= - 3.5 | C57Bl/6 | M | (Alvarez-Fischer et al., 200 Alvarez-Fischer et al., 2008 |
| **8 µg/µl** | 16 µg | 2 µl | Striatum | AP= + 0.8<br>ML= -1.9<br>DV= - 2.6 | C57Bl/6 | M | (Richter et al., 2008) |
| **8 µg/µl** | 16 µg | 4 µl | Striatum | N/A | *Not specified* | M | (Von Voigtlander & Moore, 1 |
| **1 µg/µl** | 2 µg | 2 µl | Striatum | AP= +0.9<br>ML=1.9<br>DV= -3.1 | *BALB/cyJ* | M | (Filipov *et al.*, 2002) |
| **2 µg/µl** | 4 µg | 2 µl | Striatum | AP= +0.4<br>ML=1.8<br>DV= 3.5 | C57Bl/6J | M | (Bensadoun *et al.*, 2000) |
| **3 µg/µl** | 12 µg | 4 µl | Striatum | AP=0.0<br>ML=2.0,<br>DV=-2.0 | ddY | M | (Iwata *et al.*, 2004) |
| **2 µg/µl or 4 µg/µl** | 4 µg or 8 µg | 2 µl | Striatum | AP=0.8,<br>ML=2.0<br>DV=3.6 | Mixed B6; 129S4 | M | (Perez *et al.*, 2005) |

**Table 1.1.** Parameters of mouse unilateral 6-OHDA lesion model. Adapted from (Smith & Heuer, 2011)

## 1.21 Effects of nigral grafts in the 6-OHDA lesion model

As discussed above due to the ectopic graft placement nigral grafts to not provide a circuit reconstruction as is theoretically possible in the striatal graft model. After a unilateral infusion of 6-OHDA along the nigro-striatal pathway there is a degeneration of DA neurons in the SN and subsequent depletion of DA in the striatum. This is accompanied by up-regulation of post-synaptic receptors. This asymmetry in DA release and post-synaptic receptor up-regulation leads to circling behaviour in the animal shortly after the lesion (Ungerstedt, 1968; Ungerstedt & Arbuthnott, 1970). Although there is some compensation of behaviour in animals the circling behaviour can be re-introduced via injection of drugs that act on the DA system. Amphetamine and apomorphine are the two most used compounds to study the effects of unilateral DA depletion and the effects of subsequent DA replacement via engrafting of DAergic tissue (Torres *et al.*, 2007). Interestingly, the amount of circling is related to the level of depletion in both the SN and the VTA, with circling behaviour being introduced after SN DA lesions but the amplitude of rotations depending on relative sparing of DA neurons in the VTA (Kelly, 1975; Kelly *et al.*, 1975; Kelly & Moore, 1976; Brundin *et al.*, 1987; Torres *et al.*, 2007). Next to the reduction in drug-induced rotation DAergic grafts are able to partly ameliorate the deficits on a number of tests that do not require drug administration. Small, but highly significant benefits as compared to lesioned rats were found on the stepping test, the cylinder test, and the corridor test, whilst skilled paw reaching, as assessed via the staircase test, could not be improved (Dowd *et al.*, 2005a; Torres *et al.*, 2008a). The failure to ameliorate the deficit in the staircase test is partly due to lesioned animals not attempting to retrieve pellets, rather than an inability to reach for the pellet, per se. A reduction in motivation has been reported in other tasks where lesioned rats do attempt fewer trials and make fewer responses in general, a phenomenon that has been linked to DA depletion in the nucleus accumbens (Cousins & Salamone, 1996; Dowd & Dunnett, 2004; Dowd *et al.*, 2005a; Torres *et al.*, 2008a). Also on other tests of sensory neglect DA grafts fail to improve the lesion-induced deficits in paw use, whilst improving whole body motor asymmetry in spontaneous rotation and sensori-motor function (Dunnett *et al.*, 1987). Grafts are able to improve sensori-motor and akinetic impairments, but fail to improve eating and drinking behaviour in severely, bilaterally lesioned rats (Dunnett *et al.*, 1983b). Effects of grafts are furthermore dependent on their placement within the striatum, the method of grafting, age of the donor, and depending on the function assessed (Dunnett *et al.*, 1981b; c; a; Dunnett *et al.*, 1983b; Nikkhah *et al.*, 1995), as ventro-lateral grafts can ameliorate sensori-

motor deficits whereas central grafts ameliorate drug-induced circling (Mandel *et al.*, 1990). On operant tests, 6-OHDA lesioned rats that were engrafted with VM did sustain mean lever press rates on an intracranial self stimulation task compared to rats that received cortical grafts, when the electrode was placed adjacent to the DAergic cell bodies (Fray *et al.*, 1983) and improves performance on the lateralised choice reaction time task in that engrafted rats attempt more trials, commit fewer procedural errors, improve in their contralateral response accuracy and display a reduction in their movement time latencies to execute the lateralised response, compared to lesion only controls (Dowd & Dunnett, 2004). Although a great deal of functions of DAergic grafts have been assessed and advances have been made in improving many parameters including graft survival and outgrowth, there is a lack of studies looking at recovery on more complex functions.

## 1.22 The Dowd extinction effect – "Movement without dopamine"

Recently, Dowd and Dunnett (2004, 2007) showed that animals that received near complete unilateral DA depletion showed a contralateral deficit on the Carli task that was not present when the animals were first re-introduced into the operant chambers, but instead the deficit appeared over a few days of testing (Dowd & Dunnett, 2004; 2007). Although there was near complete DA depletion, the animals' performed similarly to control animals on the first days of testing. It has been suggested that the animals initially perform on the task '*out-of-habit'* (Dowd and Dunnett, 2007, p. 429) which can temporally overcome the DA depletion. The authors argue that the observed deficit was not attributable to an impairment in motor initiation but to an impairment in reward-signalling (Dowd & Dunnett, 2004; 2007). The "extinction effect" has been linked to theories of reward signalling of neurons in the tegmental area, such as those suggested by Schultz and colleagues (Dowd & Dunnett, 2007). In a series of experiments Schultz has shown that neurons in the VTA and the SN signal the prediction of a reward in response to an external stimulus. Recordings of neurons in the VTA/SNpc in non-human primates and rats revealed that the phasic firing pattern of these neurons are able to form the neuronal basis of the 'reward prediction error' (Schultz *et al.*, 1997; Schultz, 2000; 2006). Schultz argued that the neuronal firing is not due to the presence of the rewarding stimulus itself (Schultz, 1997; 1998). The reward prediction error describes the relationship between the likelihood that a reward is obtained and the presence of the reward in a given situation. With every experience the reward prediction error is updated to

enable the animal with a prediction of a given outcome dependent on the situation and the behaviour of the animal. If the actual reward is similar to the expected reward the reward prediction error is near zero whereas if the animal anticipates a big reward and no reward is present the reward prediction error will be large. Neurons fire homogeneously when an unexpected reward is present but they inhibit firing when an expected reward is not present (Schultz *et al.*, 1992; Mirenowicz & Schultz, 1996; Hollerman & Schultz, 1998; Hollerman *et al.*, 1998; Schultz, 2001; Cromwell & Schultz, 2003). The reward prediction error forms the neuronal basis of "extinction" in the Dowd & Dunnett (2007) paradigm. Bayer and Glimcher (2005) have shown that phasic firing continues although behaviour is not adapted once the stimulus response association has been learned. Over the time course of learning the phasic response shifts from reward delivery to the onset of the first predictor or cue (Schultz *et al.*, 1997; Schultz, 2000). Interestingly, the two DAergic cell groups in the VTA and SNpc show similar firing patterns (Hollerman & Schultz, 1998; Bayer & Glimcher, 2005). It is assumed that the phasic firing of tegmental DAergic neurons towards reward predicting stimuli is needed to maintain a previously learned stimulus-response association. The lack of the phasic signal (via the lesion) leads to a reduction in responses contralateral to the lesion that resembles extinction. When animals are trained on a lateralized choice reaction time task and after training on one side is not rewarded anymore, the animal will cease responding towards that side (Dowd & Dunnett, 2007). Although the response pattern shares similarities, the pattern of decline in accuracy is less steep than the one after the lesion. After initial learning the response association is assumed to be stored at the level of plasticity at the cortico-striatal synapse (Centonze *et al.*, 2003a; Centonze *et al.*, 2003b; Calabresi *et al.*, 2006; Calabresi *et al.*, 2007). Whereas quinolinic acid lesions destroy the striatal synaptic targets of the cortico-striatal inputs, in the 6-OHDA lesion model only the supply of the phasic reward signal is broken, and not the response association itself. Neuronal replacement in the QA lesion model and in the 6-OHDA lesion model will have different effects on improving the animals' behaviour. Whereas in the QA lesion model the neural circuit that was destroyed can be reconstructed by striatal transplants because the cells are placed homotopically back into the striatum from where they are lost, in the 6-OHDA lesion model nigral cell transplants are placed ectopically, and provide local re-innervation (and DAergic activation) but do not allow for nigro-striatal reconstruction, as would be required (at least in principle) to restore reward signalling. The graft in the QA lesion model does not show a behavioural improvement after the first days of testing, but after extensive post-operative training the animals are able to "re-learn" the task (Mayer *et al.*, 1992; Brasted *et al.*, 1999b).

Because of intact reward signalling a new S-R association can be formed with the newly integrated donor tissue. Conversely, in the 6-OHDA lesion model, where the ectopic graft placement only allows tonic DA levels to be elevated, it does not allow for the reward signal to be re-constructed. Therefore, even with graft-induced improvements seen in the 6-OHDA lesion model, full recovery is unlikely to be achieved.

## 1.23 Aims of this thesis

The introduction has given a brief overview of the current state of models and research in the field of PD. Furthermore it has shown the respective level of understanding of rat and mouse models that utilise 6-OHDA to create animal models of PD. What is clear from the literature is that more work has been conducted using rat models, compared to mouse, and that not many complex behavioural tasks have been employed to investigate the effects of cell replacement therapies. With new transplantation trials fast approaching it is only a matter of time before stem cells derived from other sources will become available. It is important to know how these cells, and any cell for that matter, will function once transplanted into the brain. Whereas cell biologists are concerned with the expression of certain biomarkers, most transplantation laboratories apply rather simple (and occasionally simplistic) behavioural analyses, such as overcompensation of the rotational response to amphetamine. Although both approaches are important research tools, it is imperative to characterise the lesion model they are tested on fully, and, furthermore, to investigate whether, and to what extent, cell replacement therapies can and/or cannot ameliorate the deficits. The ultimate question will then be how do other cell sources compare to the ‘gold standard’ of primary fetal tissue.

The main goals of this thesis were to characterise the lesion-induced deficits following the 6-OHDA lesion focussing on more complex behavioural tasks of motor and cognitive function for the purpose of the assessment of cell replacement therapies. More complex analysis and exploration of what functions can be recovered and which cannot is important in the pre-clinical analysis of cell replacement therapies. Therefore, first, I aimed to utilise versions of the lateralised choice reaction time task to investigate the lesion-induced deficits and the potential of cell replacement therapies to alleviate the deficit. Second, as interest in mouse models (which are currently less well characterised) has increased over recent years, I aimed to characterise basic and more complex lesion-induced deficits in mice, followed by

an initial grafting experiment to assess the validity of the model to serve for future therapies.

Thus, my experimental programme reported in this thesis falls into a series of discrete experiments:

1.) Assessment of lesion induced deficits on choice reaction time tasks in rat models of Parkinson's disease (Experiments 1 & 3; assessed in rats)
2.) Assessment of primary fetal tissue to ameliorate lesion induced deficits on a complex behavioural task (Experiment 2; assessed in rats)
3.) Development and behavioural characterisation of mouse 6-OHDA lesion models (Experiment 4; assessed in mice)
4.) Characterisation of lesion induced deficits on complex behavioural tests (Experiment 5; assessed in mice)
5.) Assessment of primary fetal tissue to alleviate lesion induced deficits on complex behavioural tests (Experiment 6; assessed in mice)

# Chapter 2 Materials and methods

## Chapter 2.1 - Subjects

All testing was done in accordance to the UK, Scientific Procedures Animals Act of 1986 and local ethical review. All experiments were conducted under the Project Licence PPL 30/2498 and Personal Licence PIL30/8115.

### 2.1.1 Rats

In all experiments regarding rats, female animals of the Lister Hooded strain (Charles River, UK) were used. All rats were housed in standard laboratory cages in groups of 3-4. When not on food restriction the animals had free access to standard laboratory chow and water at all times. The home-cages were equipped with one cardboard tube and wood-sticks for environmental enrichment. The holding room was regulated at a temperature of 21 ± 1°C and 50% relative humidity. The light cycle was set to 12:12 hours with lights on at 07:00 am.

Rats were food restricted to 90% of their free-feeding body weights one week prior to operant training, by providing them with weighed amounts of food at the completion of each day's testing. They were allowed *ad libitum* access to water in the home cages throughout all stages of the experiment.

### 2.1.2 Mice

In experiments conducted on mice, animals of the C57/Bl6 (Harlan, UK) strain were used. Animals were held in standard laboratory cages in groups of 5-6 (Experiment 4) or 1-2 (Experiments 5 & 6). Each cage was fitted with a cardboard tube and wood-sticks for environmental enrichment. The holding room was held at a constant temperature of 23 ± 2°C and 50% relative humidity. The dark-light cycle was set to 12:12 hours with lights on at 06:00 am. Except during periods of behavioural testing in which food restriction was required all mice had access to food and water *ad libitum* at all times. During periods of testing which required motivation animals were food restricted to 85-90% of their free feeding weight by providing the animals with weighted amounts of food that was made available *ad libitum* after testing sessions. The weight of all animals was recorded three times per week and the amount of food was adjusted accordingly.

# Chapter 2.2 Apparatus

## 2.2.1 Skinner box rat

Testing in the Skinner box operant chamber (Paul Fray Ltd., Cambridge, UK) was conducted in a bank of 12 equally designed boxes (see *Figure 2.1*). The dimensions of the boxes were 26cm x 27cm x 23cm. On the front wall of the chamber two retractable levers were fitted to each side of a food magazine with a Plexiglas panel. 45mg precision sucrose pellets (Sandon, USA) could be delivered into the magazine via the attached pellet disperser. The two response levers that are located to either side of the magazine were positioned 1.5cm from the respective side wall and 6cm from the grid floor. Three stimulus lights were located 4cm above each lever and the magazine.

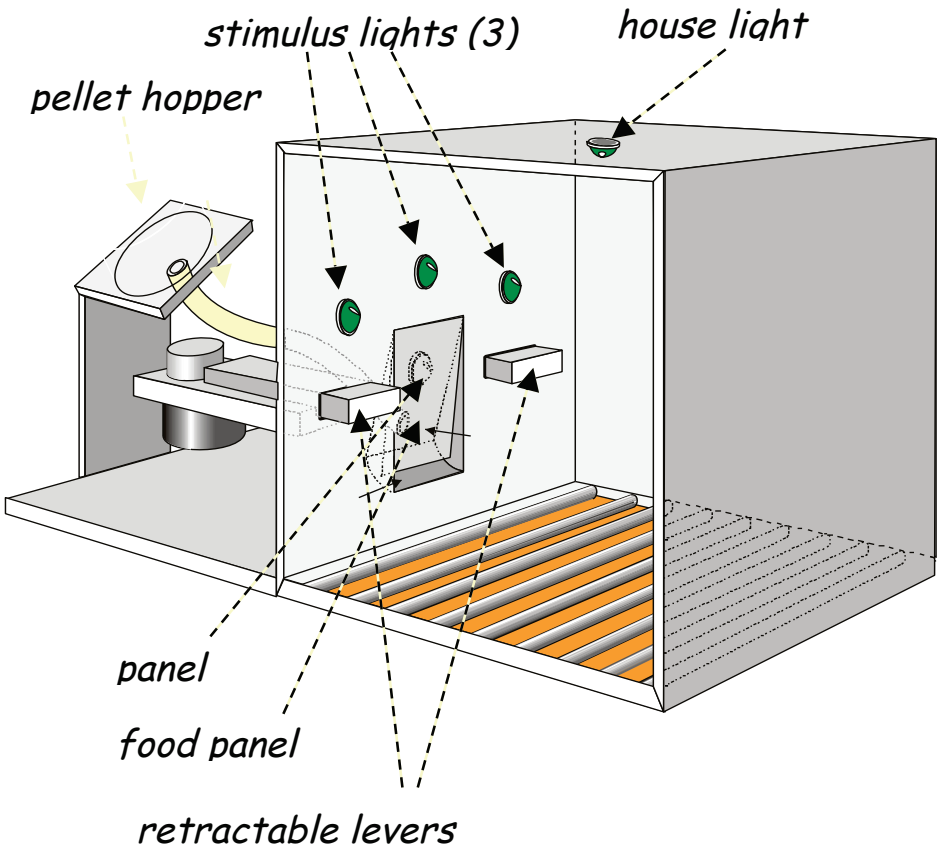


**Figure 2.1** Schematic illustration of the Rat Skinner box.

## 2.2.2 9-Hole box rat

The rat 9-hole box (*Figure 2.2)* is fitted with an array of 9 response holes mounted to the rear wall of the operant chamber which has been described previously (Carli *et al.*, 1983; Dowd & Dunnett, 2005b). Briefly, the operant chamber is fitted with a curved array of 9 response holes which can be illuminated by a green LED and detect a hole entrance via the break of a infrared beam that runs vertically across the entrance of the response hole. On the opposite side of the chamber a food magazine is fitted to which a pellet dispenser is connected. The whole apparatus is enclosed in a wooden sound attenuating cubicle with an extractor fan to dampen background noise. Holes that are not in use in a given task paradigm are typically covered with metal well blanks. The 9-hole boxes were controlled by the Cambridge Cognition Control software (Campden Instruments LTD., version 1.23) running on a standard desktop PC using the Windows XP operating system.

## 2.2.3 9-Hole box mouse

The 9-hole box used for the operant testing of mice is in principle a scaled down version of the rat 9-hole box with some changes to adapt for the smaller size of the animal. The most important changes made to the rat version is that instead of sugar pellets, strawberry milk (Yazoo®, Campina) at a volume of 5µl is provided as reward. Furthermore, the Perspex® panel door that covers the entrance of the magazine was removed and substituted by fitting infrared sensors which could detect a magazine entry (Bensadoun *et al.*, 2004).

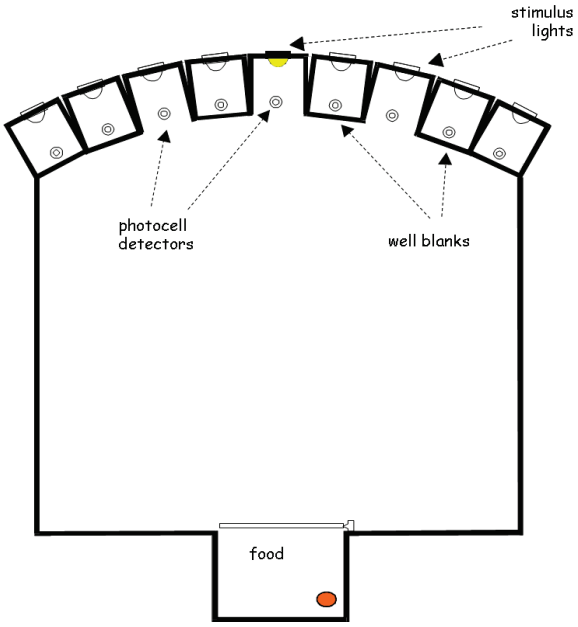


**Figure 2.2** Schematic illustration of the 9-Hole box.

# Chapter 2.3 - Operant tasks

## Task parameters

Operant tasks will be described in general and the actual parameters such as session length of stimulus duration may vary between experiments and/or between tasks that are run in rats and mice. Details of the specific task parameters can be found in the respective experimental chapter and an overview is presented in Table 2.1. Here we will focus on the general task outline only.

| Experiment | #1 Carli | #2 Brown/Brasted | #3 Error correction | #5/#6 Carli |
|---|---|---|---|---|
| Apparatus | Skinner Box | 9-Hole box | 9-Hole box | 9-Hole box |
| Species | Rat | Rat | Rat | Mouse |
| SD | 1000 | 200 | 300 | 300 |
| Holds | 100, 300, 600, 900 | 50, 100, 150, 200 | 50, 100, 150, 200 | 25, 50, 75, 100 |
| ITI | 2000 | 2000 | 2000 | 2000 |
| TOI | 5000 | 5000 | 5000 | 5000 |

**Table 2.1**. Overview of task parameters used. All times are given in ms. SD = Stimulus duration, ITI = Inter-trial interval, TOI = Time-out interval.

## 2.3.1 Carli-task in the Skinner box

In experiment 1 (Chapter 3.1) all configurations of the Skinner box version of the Carli task were adapted from Dobrossy and Dunnett (1997, 1998). The outline of the task is shown in Figure 2.3. In brief, each trial starts with the illumination of the central magazine light to which the animal has to respond with a sustained nose-pole for one of four randomly chosen delays. Upon completion of the sustained nose-poke, one of the two stimulus lights located above the response lever was illuminated (randomly determined by the computer) and signalling the correct response option. The stimulus light remains illuminated until the animal makes a response on either lever; when a lever response is made both levers are retracted. A correct response is further rewarded by the delivery of a 45mg precision sucrose pellet into the illuminated magazine. An incorrect response is ‘punished’ by a time out period in darkness. After the animal retracts its nose from the magazine after retrieving the

food reward or after the time out period a two second inter trial interval with only the house light illuminated is conducted before initiation of the next trial. The main adaptation from previous versions of the task was that the lateralised stimulus was only briefly illuminated, whereas in previous versions the light stayed on until the animal had made a response (Dobrossy & Dunnett, 1997; 1998).

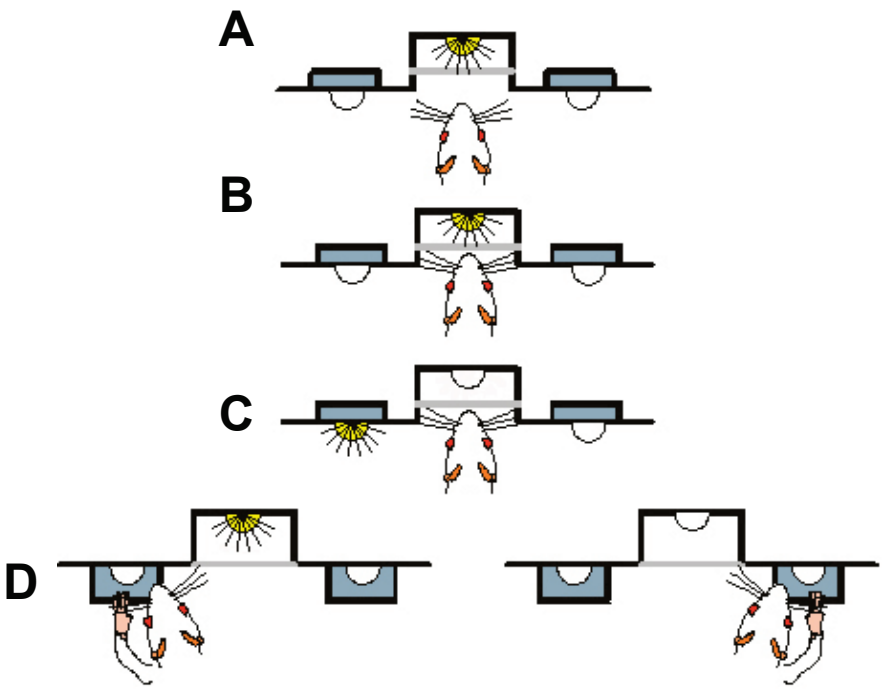


**Figure 2.3** Schematic outline of the Skinner box version of the lateralised choice reaction time task. **A.** Each trial starts with an illumination of the central stimulus light inside the food hopper. **B.** The animal is required to make a sustained nose poke for various delays (50ms, 300ms, 600ms, 900ms). **C.** If the animal does not retract its nose before the required interval, the centre light extinguishes and one of the stimulus lights located above the lateralized levers is illuminated, thereby indicating the side of required response. **D.** The animal is required to press the lever, beneath the illuminated stimulus light. A correct response (left in this example) will result in illumination of the central stimulus light and delivery of a sugar pellet into the food hopper. In case of an incorrect response (right in this example), the animal will be punished by a time out period of 5 seconds in darkness.

## 2.3.2 Brown/Brasted task in the 9-hole box

In experiments 2 and 3 we tested rats on an adaption of the classic lateralised choice reaction time task (Carli *et al.*, 1985). The current version of the choice reaction time task is adapted from Brown and Robbins (1989) and Brasted et al. (1997) and is shown in Figure 2.4. At the beginning of each trial the house lights were switched off and the centre hole was illuminated. When a nose-poke was made into the illuminated hole the centre hole light was extinguished. The animal then had to

sustain its nose-poke for a variable delay (50, 100, 150, 200ms). When the animal sustained its nose poke for the required interval one of the two lights on the left or the right (dependent on the day of testing) was randomly illuminated. Upon detection of the stimulus the rat had to retract its nose form the centre hole and perform a nose-poke in the hole indicated by the illumination. A correct response resulted in the delivery of a food pellet into the food hopper whereas an incorrect response or a premature withdrawal resulted in a 'punishment' of a time out period where all lights were extinguished (Brown & Robbins, 1989b; Brasted *et al.*, 1997).

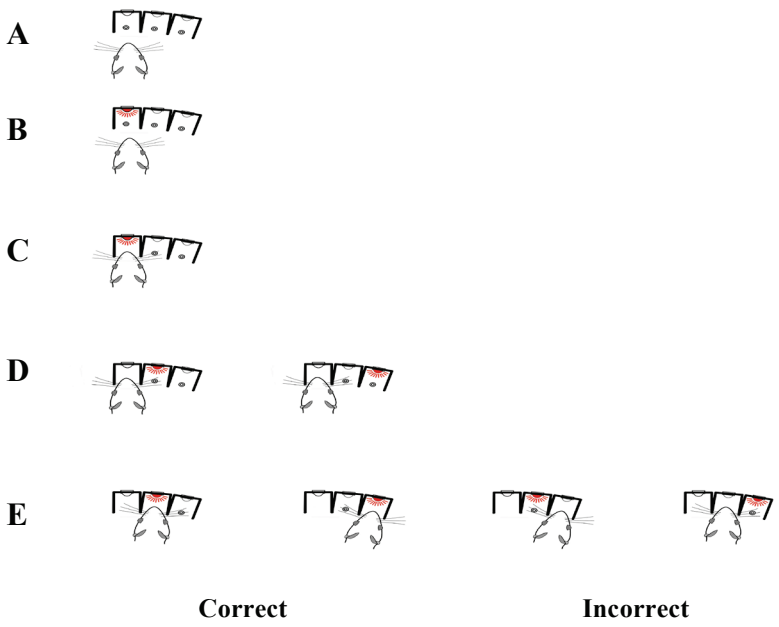


*Figure 2.4.* **A.** The animal is set in the operant chamber with only the centre hole and the two adjacent holes being exposed (right side in this example; the side is dependent on the day of testing). **B.** The stimulus light in the centre hole is illuminated. **C.** The animal has to perform a sustained nose-poke in the illuminated centre hole. **D.** One of the two stimulus lights in the adjacent holes is illuminated. **E.** A nose-poke in the illuminated hole results in reward delivery whereas an incorrect response or omission results in a time-out period.

## 2.3.3 Carli task in the mouse 9-hole box

In experiments 5 and 6 we tested mice on an adaption of the classic lateralised choice reaction time introduced by Carli et al. (Carli *et al.*, 1985; Carli *et al.*, 1989; Dowd & Dunnett, 2004). Testing was conducted in mice 9-hole boxes as described in section 2.2.3.

In brief, each trial was started with all lights extinguished, followed by constant illumination of the light in the central hole (Figure 2.5). The mouse then had to respond to the illuminated hole via a sustained nose poke into the hole for a variable

delay. Upon completion of the hold period one of the lateralised stimulus holes was briefly illuminated (randomly chosen by the computer). The mouse then had to withdraw the nose from the centre hole and respond to the hole that had been illuminated via a nose poke within a limited response time. A correct response resulted in the delivery of 5µl strawberry milk into the illuminated magazine. Upon collection of the reinforcer a brief inter trial interval was started before the next trial commenced.

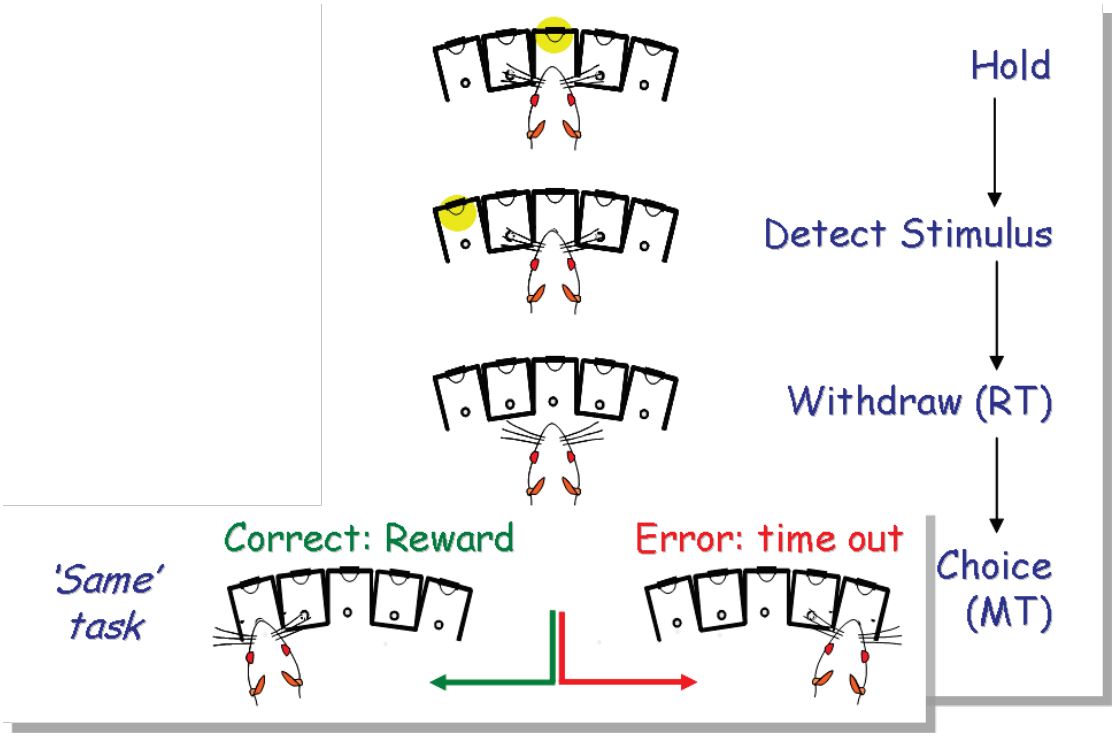


**Figure 2.5.** Outline of the lateralised choice reaction time task. After initiating a trial via a sustained nose poke into the illuminated centre hole (**A.**) the animal had to report the occurrence of a brief lateralised stimulus light (**B.**) via withdrawal of the nose from the centre hole (**C.**) and a nose poke response into the indicated response location (**D.**).

# 2.4 Simple behavioural screens

Several tests of simple motor asymmetry have been applied in the experimental chapters. A detailed description of each of the tests will be given in the respective chapter and tests will only be mentioned briefly here.

## 2.4.1 Drug-induced rotations

Drug induced rotations were induced via i.p. injection of 2.5mg/kg of methamphetamine or 0.05mg/kg apomorphine. All assessments were conducted in automated rotometer bowels which were modelled after the design of Ungersted

(Ungerstedt & Arbuthnott, 1970), with the exception of experiment 4 (Chapter 3.4), in which mice were video-recorded and rotations in either direction were scored post-hoc.

### 2.4.2 Locomotor activity

The general locomotor activity was measured in mice using Med Associate automated locomotor boxes. The animals were placed individually in plastic cages that were fitted with three infrared sensors. All beam breaks in a 2 hour period were recorded.

### 2.4.3 Cylinder test

The cylinder test measures paw use preference and utilised the exploratory behaviour of mice. Each mouse is placed in a glass cylinder and video recorded for 3 minutes from the front, with two mirrors positioned at a 60 °angle to allow for a 360° view. Post-hoc freeze-frame analysis was used to count the number of respective ipsilateral and contralateral touches of the cylinder wall.

### 2.4.4 Elevated beam

The elevated beam test measures balance and motor coordination. In brief, the mouse was placed on the lower end of an inclining beam that was wide at the base and narrowing towards the top end where a home box was located. The animal had to traverse the inclining beam to reach the home box. The number of foot slips as well as the latency to traverse the beam were scored live from one side whereas the opposite side was video-recorded and analysed post-hoc.

### 2.4.5 Corridor test

The corridor test measures sensory-motor neglect. In brief, 10 pairs of small plastic containers are placed in regular intervals along a Perspex corridor and filled with reward pellets. The mouse is placed in the corridor and allowed to retrieve pellets for 5 minutes or until 20 explorations have been made, whichever occurres first.

### 2.4.6 Staircase test

The staircase test assesses paw use and dexterity of the animal. Each staircase apparatus consists of a double staircase, which is fitted with food wells on each

individual step. Each of the steps is baited with reward pellets. Between the double staircases a platform with a plinth is located to which the mouse has to climb onto from its home box in order to reach down to the steps to retrieve the reward pellets.

### 2.4.7 Rotarod

The rotarod apparatus was used to assess general motor coordination and balance. The apparatus consists of a rotating rod whose rotation speed was increased for 4 rpm to 44 rpm over a period of 5 minutes.

### 2.4.8 Stepping test

The stepping test adapted for mice was conducted via pulling the animals backwards on a flat surface over a distance of 50 cm over 5 seconds. The mice were video-recorded from the front and the number of adjusting steps of the respective forepaw was scored post-hoc.

### 2.4.9 Gait analysis

For analysing the animals gait pattern as stride length, base width, etc., we coloured the animals' fore and hind paws with different colours and let them run over absorbent paper on a flat surface. The gait pattern of 3 consecutive strides was scored.

### 2.4.10 Grip strength

Grip strength was assessed in mice via the inverted cage lid method. In brief, mice were placed on a metal grid with a rectangle taped off. The grid was then slowly inverted and the latency to fall was recorded.

## 2.5. Surgery

General surgical protocols were identical for rats and mice. Individual differences, i.e. in lesion coordinates and toxin concentrations, are given in Table 2.1.

### 2.5.1 Lesion surgery

Anaesthesia was induced in an induction chamber with 5% isoflurane using oxygen as the carrier gas and maintained by a nose mask delivering 1-2% isoflurane in 2:1 $O_2$/NO. The surgical area was shaved and the animal was placed in a stereotaxic

frame (Kopf model 900). A 30-gauge cannula connected to a 10µl Hamilton syringe driven by a microdrive pump was used to deliver the toxin at a flow rate of 1µl/min. The required volume of 6-OHDA (see table 2.2) was calculated from freebase weight (5.14µg hydrobromide salt, Sigma Chemicals, UK) and dissolved in a solution of 0.2 mg/ml ascorbic acid in 0.9% sterile saline. After injections were made the needle was left in place for an additional 3 minutes to allow for diffusion before the injection needle was carefully retracted (Torres & Dunnett, 2007). After surgery the wound was cleaned and sutured and the animal was placed on a heated blanket for recovery. Post-operative analgesia was given by addition of paracetamol to the drinking water for three days post surgery (Experiment 1) or via s.c. injection of Metacam (Meloxicam, Boehringer, Ingelsheim, Germany).

| Species | Concentration | Dose | Volume | Target | Coordinates |
|---|---|---|---|---|---|
| **Rat** | 4 µg/µl | 12 µg | 3 µl | MFB | AP= -4.0<br>ML= -1.3<br>DV –7.0 |
| **Mouse** | 6 µg/µl | 9 µg | 1.5 µl | SN | AP= -3.0<br>ML= -1.2<br>DV= -4.5 |
| **Mouse** | 6 µg/µl | 6 µg | 1 µl | MFB | AP= -1.2<br>ML= -1.2<br>DV= -4.75 |
| **Mouse** | 6 µg/µl | 18 µg | 2 x 1.5 µl | Striatum | AP= +1.0 and +0.3<br>ML= -2.1 and -2.3<br>DV= - 2.9 and -2.9 |

**Table 2.2.** Lesion coordinates by species and target structure in mm.

## 2.5.2 Graft surgery

### 2.5.2.1 Dissection and cell preparation

Pregnant dams were deeply anaesthetized by intra peritoneal injection of sodium-pentobarbital (Euthatal) and killed by dislocation of the neck. After cleaning the abdominal area with 70% ethanol an incision was made so that the uterine horns containing the embryos could be accessed. The embryos were collected in Hank's Balanced Salt Solution (HBSS) (Gibco), and the brains were removed. The VM was dissected out as described in Dunnett and Bjorklund (1992, 1997). The dissected VMs from all embryos were pooled in one eppendorf tube and washed with HBSS. The tissue pieces were then transferred into a solution of 0.1% trypsin (Worthington, NJ, USA) and incubated for 20 minutes at 37°C. The trypsin was removed and replaced by trypsin inhibitor (Sigma Chemicals, UK) and DNAse (Sigma Chemicals, UK) and then incubated for a further 5 minutes at 37°C. The tissue was then washed in DMEM/F-12 (Gibco) and centrifuged at 1000rpm for 3 minutes to form a pellet from the tissue pieces. This pellet was re-suspended in 200µl DMEM/F-12 and

mechanically triturated with a 200µl pipette (Gilson) to produce a single cell suspension. Cell counts were conducted via the trypan blue (0.4%, Sigma Chemicals, UK) technique using a haemocytometer (Dunnett & Bjorklund, 1992; 1997).

#### 2.5.2.2 Graft surgery

Graft surgery was carried out using the same principles as lesion surgery, described in section 2.3.4.1. The main difference was that a cannula with a wider inner diameter was used to inject the cells into the denervated striatum. Detailed descriptions are given in the respective experimental chapters (Chapter 3.2 and Chapter 3.6).

# 2.6 Perfusion and histology

## 2.6.1 Perfusion

Following completion of the experiment the animals were deeply anesthetized with 200 mg/kg sodium pentobarbitone and transcardialy perfused through the heart with phosphate buffered saline (PBS) pH=7.4, followed by paraformaldehyde in PBS (Experiment 1 & 2: 4%; Experiments 3-6: 1.5%) for a duration of 5 minutes. The brains were then carefully removed from the skull and post-fixed for 24 hours in the paraformaldehyde in PBS before they were put in a 25% sucrose solution in PBS, where they were kept until they sunk (Torres & Dunnett, 2007). For visualizing lesion extent brains were sliced coronally using a freezing sledge microtome, at a thickness of 40µm. Sections were cut into 0.1 M TRIS buffered saline pH 7.4 (TBS) and stored at +4°C prior to staining. All staining was done using standard histological techniques on a 1 in 12 series of coronal sections.

## 2.6.2 Immunohistochemistry

All Immunohistochemical stains were done following the protocol of Dr. Eduardo Torres (Torres & Dunnett, 2007). A detailed list of the respective antibodies used can be found in the respective experimental chapters.

For all immunohistochemical stains coronal brain sections (40µm) were washed in TBS before quenching endogenous peroxidise activity to reduce non-specific background staining (80% distilled water; 10% $H_2O_2$; 10% Methanol) for 10 minutes. This was followed by three washes in TBS (pH 7.4) before blocking in normal serum (horse 3% in TXTBS) for 1 hour to prevent non-specific binding of the antibodies

used. After this the sections were transferred, without washing, into the primary antibody at the required concentration in TXTBS with 1% serum and incubated at room temperature overnight. After three washes in TBS the sections were then transferred into the biotinylated secondary antibody in TBS with 1% serum for 3 hours. Following this step the sections were washed again 3 times in PBS before incubation in a dako streptavidin ABC kit (Dako, Glostrupp, Denmark) in 1% serum for 2 hours. After washing three times in PBS and two washes in TNS the antibodies were visualized by staining with diaminobenzidine (DAB: Sigma, Poole, Dorset) at a concentration of 0.5mg/ml in TNS with 3% $H_2O_2$. When the colour reaction was sufficient for visualization the sections were washed 3 times in TNS and 2 times in TBS before mounting them on gelatine-covered glass slides. The slides were left to dry overnight before dehydrating in an ascending series of alcohol (70%, 95%, and 100%; 5 min each) and removal of lipids in xylene. All slides were then coverslipped.

## 2.6.3 Cell counts

After staining the sections for TH positive cells, cells of the SN were counted at the level of the medial terminal nucleus of the accessory optic tract (Dowd & Dunnett, 2004). Cell counts were done under a bright light Leica microscope with a 40x magnifying lens.

For an estimation of cell numbers in the transplants all TH-ir cells on every $6^{th}$ 40µm, thick coronal section was counted at x40 magnification (oil lens). The diameter of each cell was estimated from an average of at least 100 randomly sampled cells over all grafts of a given cell suspension group. The total number of cells per graft was estimated using the Abercrombie correction formula (Abercrombie, 1946):

$$\mathbf{T = F \times A \times M / (D/M)}$$

T = Total number of cells
F = Frequency of sections
A = Total counts for one animal
M = Section thickness
D = Average cell diameter

## 2.7 Statistical analysis

For all experiments statistical analysis used are described in detail in the respective manuscripts. In general for all experiments $\alpha$-levels of 0.05 were used to determine significant F-ratios for rejection of the null-Hypothesis.

# Chapter 3 Experimental chapters

# Chapter 3.1

## Experiment 1: Unilateral 6-OHDA lesions induce lateralised deficits in a 'Skinner box' operant choice reaction time task in rats.

PD is most commonly modelled via depletion of DA by unilateral infusion of 6-OHDA along the nirgo-striatal pathway. The injection of 6-OHDA into the MFB leads to a near-complete unilateral DA depletion, which is regarded as stable and does not undergo spontaneous recovery. Previous work conducted in the 9-hole box has shown that the lateralised choice reaction time task is sensitive enough to detect lesion-induced impairments and graft induced recovery in 6-OHDA lesioned rats. The lateralised choice reaction time task is therefore a valuable research tool to investigate the effects of unilateral manipulation of the striatum. Although a few studies have described a translation of this task to the Skinner box, no studies, to our knowledge, have used the near complete bundle lesion model.

Due to availability of operant equipment and to learn the associated procedures (surgery, programming, data analysis, histology) Professor Dunnett had me conduct the first experiment in the Skinner box apparatus.

Initial studies have shown that partially lesioned rats do recover to pre-lesion performance over two weeks of post lesion testing. In order to assess the effects of potential therapies we need to characterise the lesion-induced behavioural response profile on the Skinner box version of this task. The main questions for the present study were: (i) is the Skinner box version of the lateralised choice reaction time task sensitive to lesion induced changes? and (ii) is the deficit stable over acute and long term testing?

The experiment conducted in the present paper as well as analysis of the data, histology and preparation of the manuscript was done by the author of this thesis. Professor S.B. Dunnett was involved in planning of the experiment and gave help and advice throughout as well as in the writing of the manuscript.

Journal of Parkinson's Disease 2 (2012) 309–320
DOI 10.3233/JPD-2012-012133
IOS Press



Research Report

# Unilateral 6-OHDA Lesions Induce Lateralised Deficits in a 'Skinner box' Operant Choice Reaction Time Task in Rats

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**Abstract**.
**Background:** Parkinson's disease (PD) is most commonly modelled in rats via injection of the neurotoxin 6-hydroxydopamine (6-OHDA) into the medial forebrain bundle. Assessment of lateralised choice reaction time is usually conducted in the 9 hole box, a highly specialized apparatus that is not widely available in many behavioural laboratories. The retractable lever Skinner box on the other hand is more readily available and may have the additional advantage of faster training of animals.
**Objective:** The present study assesses the performance of lesioned rats on a lateralised choice reaction time task that allows for quantitative assessment of the behavioural profile of models of neurodegenerative diseases in the retractable lever Skinner box.
**Methods:** Here we compared the post lesion performance of pretrained female Lister hooded rats that received unilateral 6-OHDA lesions to the medial forebrain bundle and untreated controls on the choice reaction time task conducted in the Skinner box.
**Results:** Lesioned animals displayed impairments in contralateral accuracy, a reduced number of usable trials, as well as a slowing down of contralateral reaction and bilateral movement time latencies.
**Conclusions:** The findings presented allow greater comparison between laboratories, and may be useful for the investigation of treatment strategies and remedies on this model of PD.

Keywords: Parkinson's disease, 6-OHDA, operant behaviour, Skinner box, choice reaction time, dopamine, rat

## INTRODUCTION

The unilateral 6-hydroxydopamine (6-OHDA) lesion model is the most widely used model to study the dopamine loss of Parkinson's disease (PD) and to assess the ability of treatments to alleviate the symptoms [1–4]. Unilateral 6-OHDA lesions to the nigro-striatal pathway cause a degeneration of neurons in the substantia nigra (SN) and therefore a depletion of dopamine in the ipsilateral striatum on the side of the lesion. These lesions produce a sensory-motor impairment on the side contralateral to the lesion that has been well characterised on a broad range of tests of simple and more complex motor behaviours [2, 5–11]. Operant testing allows for a more in-depth analysis of the animals' behavioural change after a lesion than is allowed by simple tests of rotation, neglect or balance [7, 12]. In particular, operant reaction time tasks have been valuable in investigating the behavioural profile of models of neurodegenerative diseases [5–7, 13–31] and the effects of treatment options such as cell replacement therapy or deep brain stimulation [7, 32–39].

The lateralised choice reaction time task, first introduced by Carli et al. [5], is sensitive to dopaminergic depletion, and allows for quantitative assessment of

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ISSN 1877-7171/12/$27.50 

associated impairments in multiple aspects of the animals' simple and choice reaction and movement times [5, 6]. Usually conducted in the rat 9-hole box, rats have to report the occurrence of a brief light stimulus to either side of the animals head by responding with a nose poke into the illuminated hole. This task allows for the assessment of response accuracy as well as reaction time and movement time latencies for each side of responding [5], which is a key advantage over other reaction time measures [28] where response profiles are not separated by laterality of the stimuli and responses. The multi-level analysis of the animals' behaviour is extremely important in unilateral lesion studies where performance is not only compared between-subjects but each animal can also serve as its own 'within-subject' control. The Carli task has been shown to be a valuable tool to assess the effects of 6-OHDA terminal and bundle lesions and graft induced recovery [5–7, 22, 40].

Although the choice reaction time task has been successfully applied in the 9-hole box, this apparatus is not widely available. Therefore, in order to allow for greater comparison between laboratories, a number of studies have sought to transfer the Carli tasks to the more readily available Skinner box apparatus. In particular, the response profile of striatal lesions using quinolinic acid and ibotenic acid have been characterised in Skinner box versions of the choice reaction time task [14, 34, 37, 41], but the effects of 6-OHDA dopamine lesions has not hitherto been examined. Therefore, in the present experiment we characterise and validate the behavioural response profile of near-complete unilateral lesions of the ascending forebrain dopamine pathway on the choice reaction time task in the Skinner box, using unilateral infusion of 6-OHDA into the medial forebrain bundle (MFB), as necessary to obtain stable behavioural deficits [22].

## MATERIALS AND METHODS

All procedures carried out were in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986 and local ethical review at Cardiff University.

### *Subjects*

In the present experiment 24 female rats of the Hooded Lister strain were used (Charles River, UK), weighing 200–225 g at the time of arrival into the laboratory. They were housed in standard laboratory cages in groups of 4 with free access to water at all times. Holding rooms were maintained at an 21°C ambient temperature and 50% humidity under a 12 h:12 h dark-light cycle with lights being switched on at 7 : 00 h. Commencing 7 days after arrival and during all periods of behavioural testing, the animals were food restricted to feeding with weighed amounts of standard lab chow at the end of each daily testing session so as to maintain 90% of their free feeding weight.

### *Procedure*

A timeline of the experimental plan is presented in Fig. 1. After initial training to the boxes (see below) animals were trained on the lateralised choice reaction time task (see below). Once all rats were performing at a high level of Accuracy on the task (>90% correct trials) they were divided into two matched groups, one to receive unilateral 6-OHDA lesions ($n = 12$), the other served as sham controls ($n = 12$). The completeness of the lesions was initially assessed behaviourally, using drug-induced rotations 2 and 4 weeks after the surgery. The effect of the lesion on task performance was then assessed 5 weeks post lesion for a block of 5 consecutive days. A second assessment took place from week 17 to week 19 post lesion with two weeks under the standard configuration (week 17 & week 18; $2 \times 5$ day blocks) and one week under probe trial configuration (week 19; $1 \times 5$ day block). After completion of behavioural testing, all rats were sacrificed and the brains removed for histological analysis.

### *Apparatus*

Operant testing was conducted in a bank of 12 identical Skinner boxes (Campden Instruments, Loughborough, UK) as described previously [14]. In brief, each box was fitted with a house light, a magazine light and two stimulus lights which were located above two retractable levers, on one side of the wall. The levers were located 4 cm either side of a magazine to which 45 mg sucrose reward pellets could be delivered. The magazine entrance was covered by a hinged Perspex panel. The whole system was controlled by the Cambridge Cognition Control BNC software (Campden Instruments, Version 1.23) run on a standard desktop computer.

### *Training*

After 7 days of food restriction rats were habituated to the Skinner boxes for 30 min with 30 reward pellets placed in the lit food magazine at the beginning

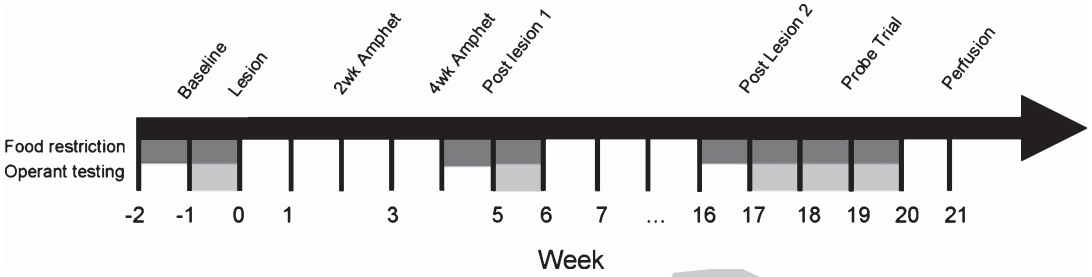


Fig. 1. Outline of the experimental plan by week of testing. Dark shaded areas indicate weeks of food restriction whereas light shaded areas indicate weeks of operant testing.

of the session. On the second day only one reward pellet was delivered in the illuminated magazine. Upon retrieval by the rat the magazine light was extinguished and the house light switched on. After a 2 s intertrial interval the magazine light was illuminated again and the house light switched off. An entry into the illuminated magazine resulted in delivery of a reward pellet. All rats earned >80 reward pellets per session over three days of training. On day 5 of training one of the retractable levers was introduced to the boxes with the stimulus light above the lever illuminated and a lever press would result in the delivery of a reward pellet. Only one lever was introduced during a given trial to prevent the animals from developing a side bias. In the next training phase, each trial was started by illumination of the magazine light and insertion of the levers into the operant chamber. A poke into the illuminated magazine would extinguish the magazine light and illuminate at random one of the two stimulus lights above the levers. A correct response (press of the illuminated lever) would result in retraction of the lever and the delivery of a reward pellet into the illuminated magazine whereas an incorrect response (press of the non-illuminated lever) resulted in a 5 s time out period with all lights extinguished. The task parameters were gradually adapted over 3 wk of testing to the settings outlined below.

*Task parameters of the lateralised choice reaction time task*

The final task parameters of the lateralised choice reaction time (Fig. 2) task were as follows: Rats were required to respond to the illumination of the magazine light by making a sustained nose poke for a variable interval (delay), randomly chosen by the computer. The four delay periods were set to 100, 300, 600, and 900 ms. Withdrawal from the magazine before the delay period elapsed resulted in a 5 s time out. Upon completion of the sustained nose poke the stimulus light above the lever was briefly illuminated for 1 s and the rat was then required to respond to the stimulus by making a lever press within 5 s.

After assessing post-lesion performance a probe trial was conducted to assess the effect of changing the stimulus duration by randomly presenting the stimulus light for 500, 1500, 2500, or 3500 ms, with all other task parameters as described above, with the delay period set to 300 ms for all trials. The main outcome measures that were collected were the number of **trials usable** (i.e. trials initiated and the nose poke sustained for the required delay period); **task accuracy** (the percentage of correct responses divided by the number of usable trials); **reaction time** (the mean latency from onset of the lateralised stimulus light to the withdrawal from the magazine); and **movement time** (the mean latency from magazine withdrawal to execution of the lateralised response).

*Drug induced rotations*

The rotational response to 2.5 mg/kg methamphetamine. HBr (Sigma, Poole, Dorset) i.p. was assessed in automated rotometer bowels modelled after the design of Ungersted [2]. Ipsilateral and contralateral rotations were recorded over 90 min commencing immediately after injection, and expressed as total average (ipsilateral – contralateral) rotation/min.

*Lesion surgery*

Inhalation anaesthesia was induced by 5% isoflurane in $O_2$ carrier gas, and maintained after mounting the animal in a Kopf model 900 stereotaxic frame by 1.5–2% isoflurane in a 2 : 1 $O_2$ : NO mixture. Lesion injections into the MFB were made by stereotaxic infusion of 3 μl of 6-OHDA HBr at a concentration of 4 μg/μl (calculated from freebase weight) in

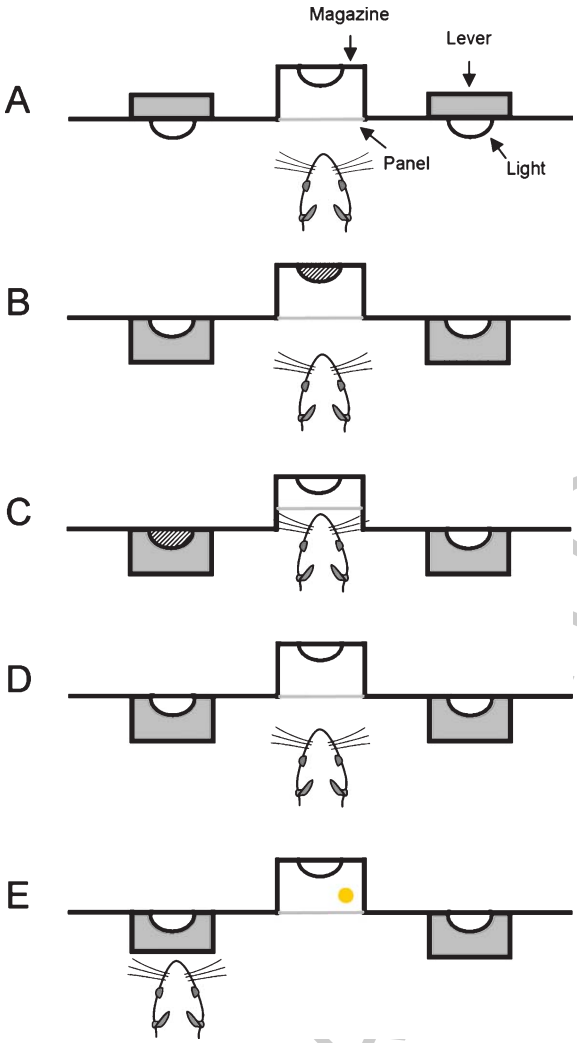


Fig. 2. Schematic outline of the operant task in the retractable-lever Skinner box used in the present experiment. (A.) Each trial starts with an inter-trial interval. After illumination of the magazine light (B.) the rat had to respond with a sustained nose poke into the magazine (C.) for the required delay. If the hold period expired, a brief stimulus above one of the levers (C.) indicated the required response. In order to obtain a food reward, the rat now had to withdraw its nose from the magazine (reaction time); (D.) and respond via a lever press (movement time); E. to the lateralised stimulus light.

a solution of 0.2 mg/ml ascorbic acid in 0.9% sterile saline via a 30-gauge stainless steel cannula targeted at: AP = −4.0 mm anterior to bregma, ML = −1.3 mm (lateral to midline), and DV = −7.0 mm (below dura). Sham lesions were conducted with the vehicle only. To control delivery, the cannula was connected via fine polyethylene tubing to a 10 μl Hamilton syringe mounted on a Harvard microdrive pump set at a flow rate of 1 μl/min and delivered over 3 min with a further 3 min allowed for diffusion of the toxin before slow retraction of the cannula. Post-lesion analgesia was given in the form of 0.3 ml injection of Metacam® (Meloxicam, Boehringer, Ingelheim, Germany) s.c. immediately after surgery.

*Histology*

Upon completion of behavioural testing, all rats were deeply anaesthetised by 200 mg/kg sodium pentobarbitone i.p. and perfused through the heart with 100 ml of 0.1 M phosphate buffered saline (PBS, pH = 7.4) followed by 250 ml of 4% paraformaldehyde in PBS. The brains were then removed from the skull and post-fixed for 24 h before being transferred into a 25% sucrose solution until they sank. The brains were cut in coronal sections (40 μm) on a freezing sledge microtome and stored in 0.1 M Tris buffered saline (TBS, pH = 7.4) at + 4°C before staining. Immunohistological staining for tyrosine hydroxylase (TH; raised in rabbit, polyclonal, Chemicon, 1 : 1000) was conducted on a 1 : 12 series of sections after a standard histological protocol [42]. The colour reaction was evoked by the stretavindin-biotin reaction with 3,3′-diaminobenzedine as chromogen [42]. After staining all sections were mounted on gelatine-coated glass slides and air dried overnight, before being cover slipped using a DPX mountant medium.

*Cell counts*

Cell numbers of TH-ir neurons were counted on a Leica DM/RBE light microscope at 10 × magnification on the side ipsilateral and contralateral to the lesion, respectively. Cell counts were conducted in one section at the level of the medial terminal nucleus of the accessory nucleus of the optic tract as an anatomical landmark to distinguish cells in the ventral tegmental area (VTA) and the SN [7]. Since the purpose was to assess relative depletion on the lesion side in comparison to the intact side (rather than to estimate actual cell numbers), no additional correction factors were applied to adjust for cell size and section thickness.

*Statistical analysis*

All data were analysed using the GENSTAT v13.1 software package (VSN International, Oxford, UK) with a significance level of $\alpha = 0.05$ chosen for all analysis. *Post-hoc* comparisons (Newman-Keuls) for significant interactions are indicated in the respective figures. Operant behavioural data were analysed by split plot analysis of variance with Group

(Control, Lesion) as between-subject factor and Side (Ipsilateral, Contralateral) and/or Week (Baseline, Lesion weeks 1–3) as within-subject factors. All latency data were analysed using geometric, rather than arithmetic, means so as to reduce the influence of infrequent very slow responses.

## RESULTS

### *Rotations*

Lesioned rats rotated at high rates towards the side of the lesion when challenged with 2.5 mg/kg methaphetamine (Group, $F_{1,44} = 81.17$, $p < 0.001$), whereas rats of the control group did not display a rotational bias. As expected, rotations were higher for lesioned animals in the second rotation test (Group × Week, $F_{1,44} = 8.63$, $p < 0.01$), with the lesion group increasing their average net rotation scores from $11.50 \pm 1.14$ to $21.37 \pm 1.87$ turns/min and the control group exhibiting only low levels of rotation at $1.05 \pm 1.31$ and $0.81 \pm 2.32$ turns/min, respectively.

### *TH-immunohistochemistry*

At the level of the striatum, the lesions induced an extensive loss of TH-ir terminals in the striatum on the injected side in all animals (Fig. 3A, B). At the level of the brainstem, the lesions caused almost complete (∼97%) loss of TH-ir cells in the ipsilateral SN and a partial (∼52%) depletion of cells in the adjacent ipsilateral VTA, whereas the cells contralateral to the lesion remain unaffected (Fig. 3C, D). Cell counts of the SN and VTA confirmed that the percentage cell depletions induced by the lesion in the SN (Fig. 3E) and VTA (Fig. 3F) were both highly significant ($F_{1,22} = 179.57$ and 83.77, respectively, both $p < 0.001$). There was no difference in cell counts between the groups in the contralateral SN or VTA ($F_{1,22} = 1.18$ and 0.16, respectively, both n.s.), confirming that the lesion effects were restricted to the hemisphere, as expected.

### *Operant task*

#### *Total trials usable*

During baseline testing all rats produced a high number of usable trials (>160) and there was no difference between the two groups (Fig. 4A, Baseline; Group, $F_{1,22} = 0.07$, n.s.). After the lesion, rats that received unilateral infusions of the neurotoxin did produce significantly less usable trials compared to their unlesioned counterparts (Control: $156.27 \pm 5.27$, Lesion: $44.22 \pm 3.67$; Week × Group, $F_{3,66} = 43.83$, $p < 0.001$). Although animals of the control group produced fewer usable trials during the last two weeks of testing, the effect of the lesion was stable over the three time points of post-lesion assessment (Week × Group, $F_{2,44} = 2.68$, n.s.).

#### *Accuracy*

During baseline testing there was no difference between rats of the control and rats of the lesion group as they were matched according to this outcome measure (Fig. 4B; Control: $97.01\% \pm 0.23$, Lesion: $95.99\% \pm 0.36$; Group, $F_{1,22} = 1.30$, n.s.). All animals performed with a high accuracy to stimuli on each side (Sides, $F_{1,22} = 0.26$, n.s.). After receiving unilateral infusion of 6-OHDA lesions to the MFB, the lesion groups' accuracy was significantly reduced compared to rats of the control group (Control: $95.63\% \pm 0.27$, Lesion: $46.21\% \pm 1.94$; Week × Group, $F_{3,66} = 44.05$, $p < 0.001$). Whereas control rats continued to perform with high accuracy to stimuli presented to either side of the animals' head, lesioned rats performed with a decreased accuracy to ipsilateral and contralateral presented stimuli, with the contralateral side affected more (Sides × Groups, $F_{1,22} = 28.18$, $p < 0.001$). The deficit at the 17 wk post-lesion time point was similar to the 5 wk post-lesion testing period (Week, $F_{1,22} = 1.68$, n.s.), with a significantly reduced response accuracy in the lesion group which was more pronounced on the side contralateral to the lesion (Side × Group, $F_{1,22} = 19.75$, $p < 0.001$). Previous reports have shown an 'extinction' like effect during the first days of testing when rats are re-introduced into the operant chambers. Although rats of the lesion group responded with a higher overall response accuracy on the first day of testing after the lesion (Day 1: $35.01\% \pm 4.39$) compared to last day of testing in the post lesion block (Day 5: $18.05\% \pm 4.57$), this difference failed to reach statistical significance (Days × Group, $F_{4,88} = 2.28$, $p = 0.067$). After re-introducing the rats into the boxes at week 17 post lesion showed the same effect with none of the days of testing being significantly different from each other (Day × Group, $F_{4,88} = 2.11$, n.s.).

#### *Reaction time*

At time of baseline testing there was no difference between the groups on the time taken to detect the lateralised stimulus (Fig. 4C; Control: $0.31\ s \pm 0.01$, Lesion: $0.31\ s \pm 0.01$; Group, $F_{1,22} = 0.00$, n.s.). After the lesion animals of the lesion group responded slower when the stimulus was presented on the side contralateral to the lesion (Contralateral: Control: $0.31\ s \pm 0.01$,

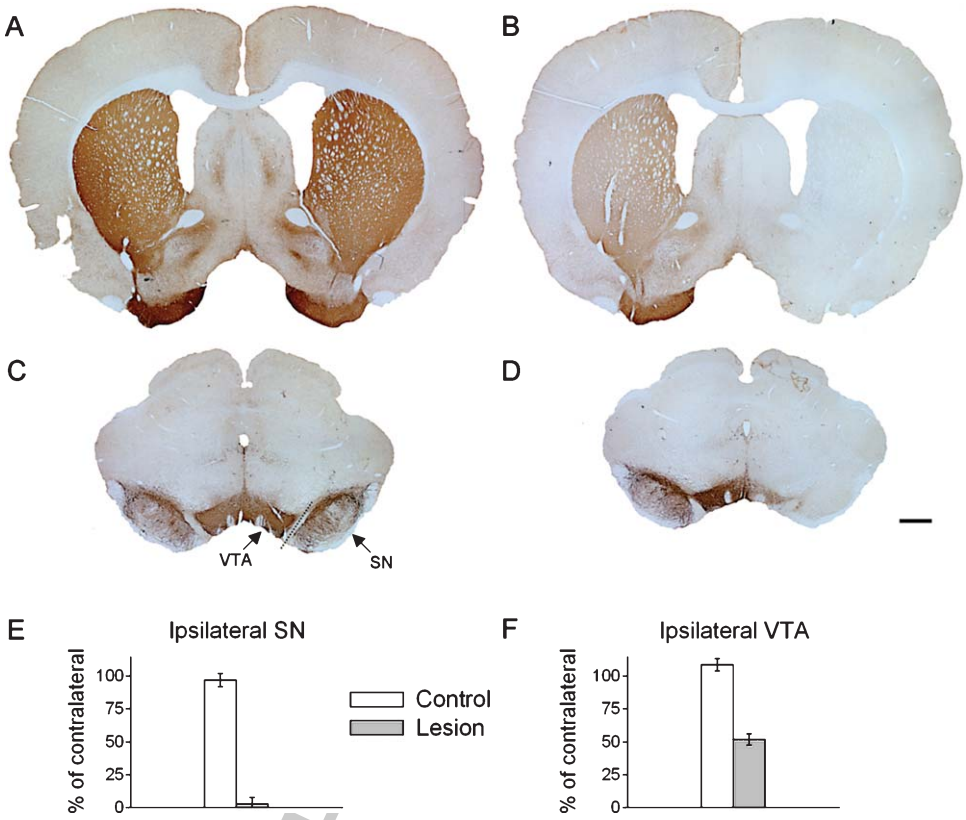


Fig. 3. Histological confirmation of the lesion. Representative samples of coronal sections stained for TH for Control (A, C) and Lesion (B, D) groups. Note the lack of TH-ir staining in the ipsilateral striatum and the loss of TH-ir cell bodies in the ipsilateral SN. Scale bar represents 1000 μm. Cell counts of TH-ir cells in the midbrain at the level of the accessory optical nerve for neurons in the SN (E) and VTA (F), respectively. Cell counts are expressed as percentage remaining cells ipsilateral from total per experimental group.



Lesion: 0.49 s ± 0.04; Group × Sides, $F_{1,22} = 13.34$ $p < 0.001$). The difference between the groups did not undergo spontaneous behavioural recovery even after testing the animals 12 weeks later (wk 5 vs. wk 17; Weeks × Groups, $F_{1,22} = 0.61$, n.s.) and lesioned animals continued to respond with prolonged reaction times to contralateral stimuli (wk 5 vs. wk 17; Sides × Group, $F_{1,22} = 34.85$, $p < 0.001$). Furthermore, even with additional continuous testing there was no recovery on reaction time between the second chronic week of testing and the other two post-lesion assessments (Weeks × Groups, $F_{1,44} = 1.26$, n.s.).

*Movement time*

During baseline data acquisition, there was no difference in the time to execute the lateralised response between the groups (Fig. 4D; Control: 0.72 s ± 0.01, Lesion: 0.75 ± 0.01; Groups, $F_{1,22} = 0.10$, n.s.) nor was there a difference in response speed between the sides of stimulus presentation (Sides, $F_{1,22} = 0.00$, n.s.). After the lesion, movement times were prolonged in the lesion group compared to animals of the control group (Contralateral: Control: 0.70 s ± 0.02, Lesion: 1.94 s ± 0.10; Ipsilateral: Control: 0.73 s ± 0.02, Lesion: 1.56 s ± 0.06; Weeks × Group, $F_{3,66} = 29.71$, $p < 0.001$). Although lesioned animals displayed prolonged movement latencies towards both sides compared to control animals, they took significantly longer to respond towards the side contralateral to the lesion (Weeks × Sides × Group, $F_{3,66} = 11.65$, $p < 0.001$). The prolonged movement time latencies were stable in the lesion group over the three time points of assessment with contralateral response times even increasing over the course of testing (Weeks × Sides × Group, $F_{3,44} = 6.78$, $p < 0.01$).

*Errors*

Procedural errors, in the form of premature withdrawals from the magazine, were the most common type of errors made (Table 1). These accounted for 25% of all aborted trials that were initiated. Although the error rate increased over the course of testing from

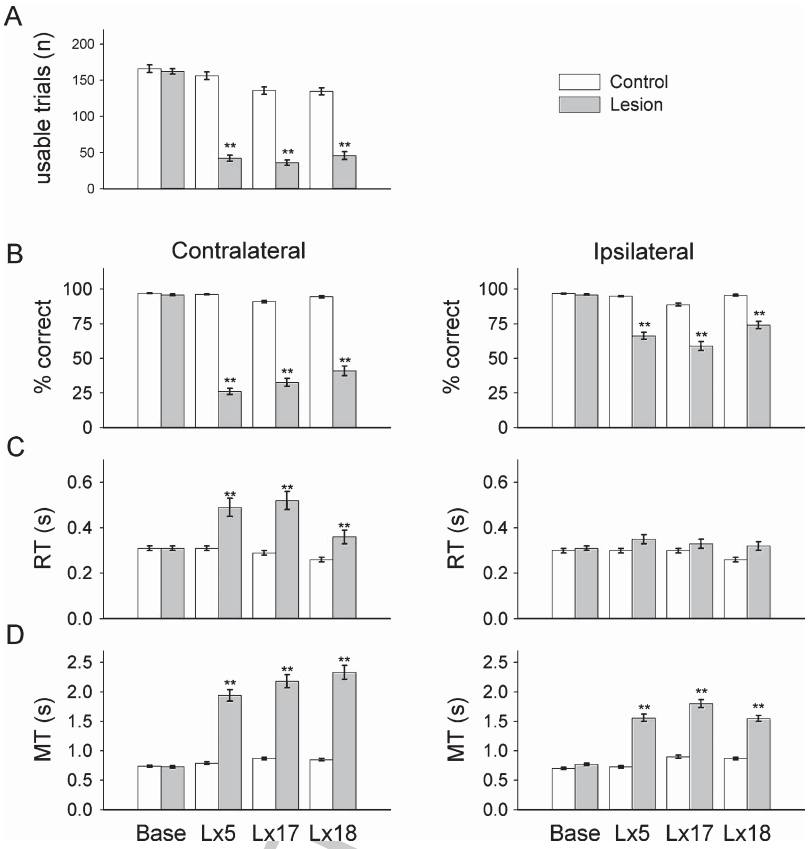


Fig. 4. Main Parameters of the lateral choice reaction time task presented by week of testing and side. Lesioned rats produced fewer usable trials A. and displayed a lower response accuracy B., especially when the required response had to be directed to the side contralateral to the lesion. The time to react to the contralateral stimulus light via withdrawal of the nose from the magazine was increased in lesioned animals for contralateral stimulus presentations C. and the latencies to execute the lateralised response was increased in lesioned animals D., with latencies to the contralateral side to the lesion being slower than ipsilateral responses. Asterisks denote significant different at the $p < 0.01$ level of significance.

22% at baseline to 28% during the last week of operant testing (Week, $F_{3,66} = 3.41$, $p < 0.05$), the lesion did not affect premature withdrawals (Week × Group, $F_{3,66} = 0.94$, n.s.). In contrast, lesioned animals made significantly more errors in the form of lever responses instead of initiating the trial by a nose poke into the magazine (Week × Groups, $F_{3,66} = 7.65$ and 7.38 for the ipsilateral and contralateral levers, respectively, both $p < 0.001$). Lesioned animals missed more lateralised responses after completion of the sustained nose poke. Approximately 10% of trials started were not completed by a lateralised response in lesioned animals within the 5 s of limited response time, compared to 1% of trials in the control group (Week × Group, $F_{3,66} = 12.63$, $p < 0.001$).

*Probe trial*

After assessing chronic post-lesion performance, we manipulated the stimulus duration to assess the effects of reduced task difficulty. In the present paradigm the stimulus duration was randomly selected by the controlling computer to be either 500 ms, 1500 ms, 2500 ms, or 3500 ms. Note that the 500 ms duration was shorter than the duration used in the standard setup of the task (1000 ms) whereas the longest stimulus duration was longer than rats of the lesion group required to execute the lateralised response on the most impaired side (2300 ms). The longer stimulus duration was thought to reduce the cognitive workload as rats to not have to remember the location of the lateralised stimulus until the response is executed; rather they could have used the stimulus light as guidance towards the response location.

All rats responded on the manipulated version of the choice reaction time task as during the standard configuration. Similar to the standard configuration, lesioned rats produced a lower number of usable trials compared to their unlesioned counterparts (Fig. 5A;

Table 1
Procedural errors on the choice reaction time task

| | Week | Control | | Lesion | | *Post-hoc* |
|---|---|---|---|---|---|---|
| Premature withdrawal (% of TTS) | Baseline | 21.95 | ±1.29 | 22.87 | ±0.95 | n.s. |
| | L × 5 | 24.02 | ±1.42 | 30.05 | ±1.42 | $p<0.05$ |
| | L × 17 | 25.93 | ±1.52 | 25.50 | ±1.42 | n.s. |
| | L × 18 | 28.51 | ±1.28 | 28.67 | ±1.62 | n.s. |
| Lever press instead of centre poke (% of TTS) | Baseline | 1.08 | ±0.15 | 1.27 | ±0.23 | n.s. |
| | L × 5 | 1.00 | ±0.11 | 25.38 | ±2.11 | $p<0.01$ |
| | L × 17 | 3.66 | ±0.44 | 32.88 | ±2.48 | $p<0.01$ |
| | L × 18 | 1.34 | ±0.18 | 30.18 | ±3.03 | $p<0.01$ |
| Omission of lateralised response (% of TTU) | Baseline | 2.46 | ±0.30 | 2.88 | ±0.35 | n.s. |
| | L × 5 | 3.10 | ±0.28 | 20.25 | ±1.61 | $p<0.01$ |
| | L × 17 | 4.16 | ±0.67 | 24.40 | ±2.29 | $p<0.01$ |
| | L × 18 | 3.16 | ±0.44 | 27.91 | ±2.16 | $p<0.01$ |

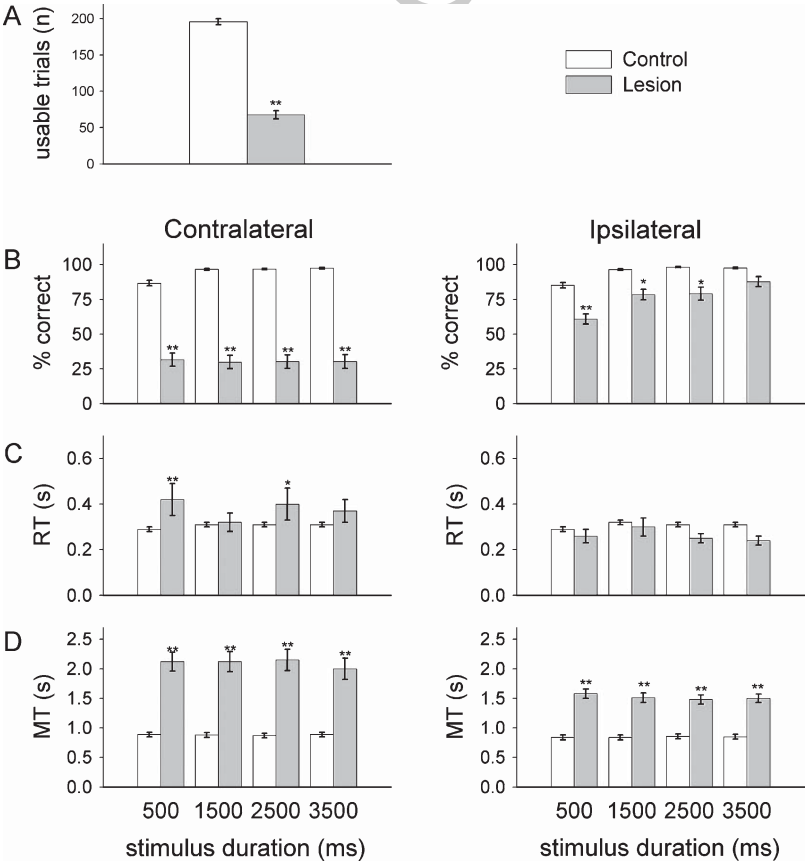


Fig. 5. Main Parameters of the lateral choice reaction time task for the probe trial, presented by stimulus duration and side. Lesioned rats produced fewer usable trials A. and displayed a lower response accuracy B., especially when the required response had to be directed to the side contralateral to the lesion. On ipsilateral days of testing increasing the stimulus had a positive effect on response accuracy in lesioned animals. The time to react to the contralateral stimulus light via withdrawal of the nose from the magazine was increased in lesioned animals for contralateral stimulus presentations C. but was not affected by the stimulus duration. Latencies to execute the lateralised response were increased in lesioned animals D., with latencies to the contralateral side to the lesion being slower than ipsilateral responses. The number of asterisks denote significant differences at the $p<0.05$ and $p<0.01$ level of significance, respectively.

Group, $F_{1,22}=34.35$, $p<0.001$). As previously, response accuracy was significantly different between the two groups whereby the lesioned rats respond with a reduced response accuracy on both sides, whereby accuracy is reduced much more when a response was required on the side contralateral to

the lesion (Fig. 5B; Sides × Groups, $F_{1,22} = 18.34$, $p < 0.001$). Stimulus length was significantly affected accuracy with longer stimulus durations resulting in a higher accuracy (Duration, $F_{3,66} = 15.31$, $p < 0.001$). Note that the shortest stimulus duration was shorter that during the standard setting. Manipulating the stimulus duration had a different effect depending on the side to which the response required was made (Duration × Sides, $F_{3,66} = 5.39$, $p < 0.001$), with the ipsilateral side responses displaying a larger increase in response accuracy with increasing stimulus length (Ipsilateral; Duration, $F_{3,66} = 7.43$, $p < 0.05$), which was not different between the groups (Ipsilateral; Duration × Group, $F_{3,66} = 1.27$, n.s.), when compared to the side contralateral to the lesion. Here only the control group benefitted from the increased stimulus duration whereas the lesion group did respond with equal accuracy to either of the four stimulus lengths (Contralateral; Duration × Group, $F_{3,66} = 5.35$, $p < 0.01$). As during the other post-lesion time points reaction times were increased for lesioned rats on the contralateral side (Fig. 5C; Sides × Group, $F_{1,22} = 13.70$, $p < 0.01$) but reaction times were not affected by the duration of stimulus presentation (Duration × Group, $F_{3,66} = 1.24$, n.s.). The time to execute the lateralised response was similar for all four stimulus durations (Fig. 5D, Duration, $F_{1,22} = 0.26$, n.s.) and, as previously, lesioned rats responded slower to both sides with a more pronounced deficit when responses were made to the side contralateral to the lesion (Sides × Groups, $F_{1,22} = 7.78$, $p < 0.05$).

## DISCUSSION

In the present experiment we confirm that the Skinner box apparatus offers the same experimental utility for assessing lateralised reaction time impairments in experimental hemiparkinsonian rats, and does not require use of a specialist 9-hole box facility. Specifically, we have assessed the effects of unilateral near-complete dopamine depletion on the lateralised choice reaction time task in the Skinner box in rats, and shown that the lesion caused impairments in response accuracy that were more pronounced when responses had to be made on the side contralateral to the lesion, accompanied by increases in contralateral reaction time latencies and bilateral movement time latencies, with response execution towards the contralateral lever being more impaired. The lesion induced deficits were relatively stable, being similar in sub-acute (5 wk post-lesion) and chronic (17–18 wk post-lesion) testing.

Near-complete unilateral lesions on the lateralised choice reaction time task produce similar deficits as have been reported when testing was conducted in the 9-hole box apparatus [22, 23]. The major differences were that in the 9-hole box the deficit was more lateralised, with response accuracy not being affected on the ipsilateral side of the lesion [7, 23], whereas in the present experiment there was a 20% reduction in accuracy on the ipsilateral side also. However the contralateral deficit was large, stable over the course of testing, and could not be alleviated by the reducing task demands. Furthermore, the lesions caused a marked reduction in the number of usable trials which we consider most likely to be attributable to reduced motivational state, since these lesions caused a significant depletion in the VTA and the ventral striatum/nucleus accumbens [43] in addition to the destruction of TH-ir cells in the SN. The differences in response profile are likely to be due to differences in the configuration of the different forms of operant apparatus. Although the tasks are conceptually the same, in the 9-hole box the lateralised response is made via a nose poke by the rat into the previous illuminated response hole, whereas in the Skinner box a lever press is required. Depending on the distance from the centre hole, the lateralised response is one continuous event in the 9-hole box, whereas a series of movements are required for the execution of the lever press in the Skinner box [44]. These differences are represented in the prolonged movement times in the Skinner box (here: 1.5–2 s), whereas the response in the 9-hole box is shorter than 1 s [7, 22]. Indeed, even in the 9-hole box initial studies did not find an increase in contralateral movement time when the response hole was immediately adjacent to the centre hole [5], whereas when the response hole was moved further away from the centre location stable movement time deficits were found [22, 23]. Rats need to use all parts of their body to execute the ipsilateral response in the Skinner box setting, including the side that is controlled by the lesioned hemisphere. We therefore speculate that the apparatus revealed ipsilateral deficits because of the nature of the response required, whereas the ipsilateral response in the immediate adjacent hole in the 9 hole box does require relatively little movement [5].

During post-lesion assessment we observed the animals during the task and discovered that the increased movement time latencies were not only due to a general slowing of movements but rather due to a change in response strategy. As has been reported previously in the Skinner box version of the task [14], lesioned rats respond to the contralateral lever by making a full

360° body turn, a strategy that has not been described when testing was conducted in the 9-hole box [5, 22, 23, 29, 44].

Interestingly, increasing the stimulus duration for longer than the contralateral response took to execute (∼1500–2000 ms) did not increase response accuracy in lesioned rats. This suggests that they are not simply attending to the ipsilateral stimulus after they exit the magazine and responding towards the ipsilateral lever if the stimulus light is illuminated, whereas they continue to turn and press the contralateral lever if the stimulus light is not illuminated. If this were the case, we would have expected more incorrect ipsilateral responses at the shortest stimulus duration (500 ms) than at the longest stimulus durations (3500 ms), which was not the case. Whereas ipsilateral response accuracy increased for both groups with increased stimulus length, contralateral response accuracy was equally unaffected in lesioned rats for short and long stimulus durations.

In earlier Skinner box studies, assessing striatal lesions [14], it was speculated that the response could be broken down into three separate events: (i) lateralised attention, (ii) withdrawal from the magazine after stimulus detection, and (iii) execution of the lateralised response [14]. Furthermore, we postulated that lateralised attention was not necessary in the Skinner box version during the sustained magazine press as the stimulus was illuminated until the rat made a lateralised response (or had been timed out), where as it was imperative in the 9-hole box as the stimulus was only presented briefly (200 ms). Here we show that the response deficit, in unilateral 6-OHDA near-complete lesioned rats, is similar for both conditions, i.e. (i) when the stimulus duration is short and lateralised attention is necessary, and (ii) when the stimulus duration is long and it is not necessary.

When lateralised reaction time was assessed in the 9-hole box with similar lesion an interesting effect was observed. When lesioned rats were first re-introduced to the operant boxes the deficit in response accuracy was not apparent on the first day of testing but gradually emerged over the following days. This effect was similar when rats were re-introduced 2 weeks post lesion as well as 12 weeks post lesion [7, 45]. This extinction profile was similar to when one of the sides is not rewarded [45]. The authors argue that dopamine is not necessary to complete the contralateral movement, but rather that it is necessary to learn and maintain stimulus-response associations [45]. Although response accuracy was declining over the first 5 days of re-testing animals at the first time-point after the lesion, this decline failed to reach statistical significance. Furthermore, even on the first day of re-testing lesioned rats displayed a deficit in response accuracy in the Skinner box apparatus, whereas lesioned rats did not display a deficit on day 1 when testing was conducted in the 9-hole box [7, 45]. This difference is likely to be the result of differences in the apparatus and the nature the lateralised response as executed. As described above, the response in the Skinner box requires a greater movement pattern, and can be broken down into more steps, whereas the response in the 9-hole box is relatively simple, i.e. it does not require additional use of the paw to respond to the manipulandum. It would be interesting to test animals in the 9-hole box with the response options moved further away from the centre location to see if this is the case. A difference in lesion extent/remaining dopamine is unlikely as in both types of studies lesions are almost complete (present manuscript: SN: 97% depletetion; Dowd & Dunnett 2005, 2007 : 98% and 96% depletion, respectively).

Although the first testing session in the present experiment was conducted 5 weeks after the lesion whereas in previous reports testing commenced already two weeks after the lesion, the time difference is unlikely to have and effect as the same pattern of 'extinction' was seen during acute (2 week post lesion) and chronic (12 weeks post lesion).

The validation of the lateralised choice reaction time task in the unilateral 6-OHDA lesion model will enable a more widespread comparison of behavioural data, as the Skinner box is more widely available across behavioural laboratories. Although the 9-hole box has many advantages, the retractable levers in the Skinner box allow a response option to be withheld from the animals, thereby focussing them on the task, whereas intrusive response options cannot be withheld in the 9-hole box. This might lead to a faster training and fewer errors committed [14]. Operant analysis of behaviour is particularly valuable for the screening of therapeutics and for investigation in the underlying deficits of any disorder, not least because of the objective collection of automated data, the large numbers of trials that can be run under standardised conditions to reduce variability and enhance the power to estimate small effects, and the ability to collate a comprehensive profile of behavioural function from multiple simultaneously collected parameters of performance. Future work can now be addressed to investigate the effects of cell replacement therapies or other therapeutics on this lesion model, as had been done in the 9-hole box [7, 34, 39, 40].

## CONCLUSION

We here reported the effects of near-complete unilateral lesion model of PD on a lateralised choice reaction time task in the Skinner box. 6-OHDA lesions induced deficits comparable with previous reports which have been conducted in the 9-hole box in a conceptually similar task. The lesion induced a stable deficit that involved a reduction of usable trials, a reduction of response accuracy when the conditioned response had to be directed in contralateral space as well as increased contralateral reaction and bilateral movement time latencies. The present design is therefore useful for the investigation of lesion-induced deficits and long term testing of therapies, without the risk of spontaneous recovery. The validation of the near complete unilateral 6-OHDA lesion model on the Skinner box version of this task allows for a wider comparison of results between behavioural laboratories.

## ACKNOWLEDGMENTS

The work has been supported by funding from the European Community's Seventh Framework Programme, NeuroStemCell (grant agreement nr. 222943) and the Medical Research Council.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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# Chapter 3.2

## Experiment 2: Dopamine-rich grafts alleviate deficits in contralateral response space induced by extensive dopamine depletion in rats

The present study was inspired by two strands of research: (i) studies have shown that 6-OHDA lesioned rats only display a severe contralateral neglect when tested in a bilateral hole configuration but when tested only on the side contralateral to the lesion, in a choice reaction time setting with a proximal and a distal response location, they are perfectly able to respond in contralateral space, but rather bias their responses towards the proximal response location on the contralateral side; and (ii) a conceptually similar task has been used to investigate the effects of cell replacement therapy in a quinolinic acid lesion model of HD. This and other studies have shown that the transplant is not effective in ameliorating the lesion induced response bias, but rather a period of specific training is required.

The effects of primary VM transplants in the rat model of PD using the choice reaction time task has been reported previously as has the spatial version of the task using terminal lesions. The novel adaptation of the Carli task allows for the assessment of more parameters as well as a more complex analysis of the spatial neglect that is induced by the lesion. Although the Carli task has been shown to provide valuable information on the restorative capabilities of primary fetal tissue we here wanted to explore the capabilities and limitations of the same tissue on the novel adaptation of this task. For this we firstly need to demonstrate that bundle lesions produce a stable and lasting deficit that does not undergo spontaneous recovery, i.e. mask the potential therapeutic effect, and show the long term effect of repeated testing.

The experiment conducted in the present paper as well as analysis of the data, histology and preparation of the manuscript was done by the author of this thesis. Professor S.B. Dunnett was involved in planning of the experiment and gave help and advice throughout as well as in the writing of the manuscript. Dr. Torres dissected the embryos and prepared the cell suspensions and Dr. Kelly helped with the transplantation of the tissue. Dr. Lelos, Dr. Kelly and Dr. Torres provided advice throughout and gave input into the final version of the manuscript.

Experimental Neurology xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Regular Article

# Dopamine-rich grafts alleviate deficits in contralateral response space induced by extensive dopamine depletion in rats

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ARTICLE INFO

*Article history:*
Received 27 September 2012
Revised 7 January 2013
Accepted 21 January 2013
Available online xxxx

*Keywords:*
Parkinson's disease
Striatum
6-Hydroxydopamine
Operant behaviour
9-Hole box
Choice reaction time
Spatial
Egocentric
Graft
Ventral mesencephalon
Primary fetal tissue

ABSTRACT

Unilateral infusion of 6-hydroxydopamine into the nigro-striatal pathway in the rat is the most common dopamine lesion model of Parkinson's disease. In the present study, we explore the impact of near complete unilateral loss of dopamine along the nigro-striatal pathway and subsequent cell replacement therapy in a choice reaction time task in rats, with assessment of spatial responding towards either side of the body (ipsilateral or contralateral to the lesion) on alternate days. Results indicated a stable contralateral deficit in response accuracy, reaction times and motor function for 50 consecutive days of testing, with no signs of recovery or compensation. All lesioned rats developed a near-hole bias and displayed prolonged movement and reaction times when responses had to be directed towards a distal response location on the side of the body contralateral to the lesion, as well as a smaller ipsilateral impairment in response accuracy and movement times. Grafts of dopamine-rich tissue into the denervated striatum improved some, but not all, of the deficits induced by the lesion. Specifically, grafted rats performed at a similar level to control animals when assessed on the ipsilateral side, they demonstrated a partial restitution of their ability to respond to far contralateral stimuli, and they exhibited a marked reduction in the time to complete all lateralised responses on both sides. The present characterisation of the task and the effects of cell replacement via primary fetal mesencephalic tissue demonstrate restorative properties in alleviating the marked spatial response bias induced by unilateral loss of dopamine.



## Introduction

To model Parkinson's disease (PD) in the laboratory, loss of dopamine cell projections from the SN to the striatum is typically induced in the rat using the unilateral 6-hydroxydopamine (6-OHDA) lesion (Bove et al., 2005; Deumens et al., 2002; Grealish et al., 2008; Kirik et al., 1998; Ungerstedt and Arbuthnott, 1970). This results in a 'hemiparkinsonian' syndrome with degeneration of dopaminergic cells on the ipsilateral side of the toxin injection. Behaviourally these unilateral lesioned rats will display impairments on the side opposite (contralateral) to the side of toxin injection. Specifically, lesioned rats display marked impairments in detecting and responding to visual stimuli (e.g. lights) on the contralateral side of the body (Brown and Robbins, 1989a, 1989b; Carli et al., 1985, 1989; Dowd and Dunnett, 2004, 2005a, 2005b; Ljungberg and Ungerstedt, 1976). A characteristic behavioural response profile of such a lesion can be found when unilateral lesioned rats are tested in a choice reaction time task conducted in the 9-hole operant chamber, in which rats have to report the occurrence of a lateralised stimulus light presented to either side of the animals' head. This paradigm reveals a characteristic response bias, which manifests as impaired accuracy, reaction and movement times when responses are directed to the side contralateral to the lesion, irrespective of the side of stimulus delivery (Carli et al., 1985, 1989; Dowd and Dunnett, 2004, 2005a, 2005b). This bias to respond preferably to the ipsilateral side of the lesion was characterised further by Brown and Robbins (1989a) who have shown that lesioned animals were able to respond accurately in contralateral space, but developed a response bias to a proximal response location when offered a choice between two response options (Brown and Robbins, 1989a, 1989b). The authors argue that dopamine depletion results in a distortion of spatial coding when responses have to be directed into a spatial location on the contralateral side of the lesion while remaining unimpaired when responding towards a spatial location on the ipsilateral side (Brown and Robbins, 1989a, 1989b).

To replace dopamine innervation in denervated target areas, cell replacement therapies have been developed involving transplantation of dopamine rich tissue harvested from the developing fetal ventral mesencephalon (VM) to provide a long-term restitution of synaptic DA release in the denervated striatum (Björklund et al., 1980, 2003; Perlow et al., 1979). Ectopic engraftment into the denervated striatum has shown to be a promising intervention to alleviate certain aspect of the impairment induced by 6-OHDA lesions in rats (Björklund and Stenevi, 1979; Dowd and Dunnett, 2004; Dowd et al., 2005; Dunnett and Bjorklund, 1999; Fray et al., 1983; Torres et

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http://dx.doi.org/10.1016/j.expneurol.2013.01.020

al., 2008). Detailed analysis of lesion-induced impairments is imperative for the development and assessment of therapeutics for PD. Whereas on the lateralised version of the choice reaction time task the effects of the lesion were assessed long term, on the novel version introduced by Brown and Robbins (1989a, 1989b) the stability of the deficit is unknown and spontaneous recovery might impede the usefulness of the task to assess therapeutic interventions (Dowd and Dunnett, 2004, 2005a, 2005b). With an increase in the development of alternative cell sources to primary fetal tissue (Kriks et al., 2011), it is imperative not only to provide a detailed analysis of behavioural deficits induced in animal models of the disease, but also to fully characterise the parameters that can be improved by a given intervention. Choice reaction time tasks have been valuable in characterising response profiles induced by lesions (Baunez et al., 1995a, 1995b; Brasted et al., 1997; Brown and Robbins, 1989a, 1989b; Carli et al., 1985, 1989; Courtiere et al., 2005; Döbrössy and Dunnett, 1997; Dowd and Dunnett, 2005a, 2005b; Ward and Brown, 1996), to probe pharmacological challenges (Baunez et al., 1995a, 1995b; Blokland, 1998; Blokland et al., 2005; Scholtissen et al., 2006), assess transgenic animals (Fielding et al., 2012; Temel et al., 2006), test cell replacement therapies (Brasted et al., 1999a, 1999b, 2000; Dowd and Dunnett, 2004) and evaluate deep brain stimulation (Darbaky et al., 2003; Temel et al., 2005, 2006).

Although the acute effects of dopamine depletion have been tested after terminal lesions 5 days and 4 weeks post-lesion, no long-term assessment of the stability of the lesion has been reported. Stable lesions are imperative for the assessment of cell replacement therapies as there is a risk of spontaneous recovery with incomplete lesions due to compensatory mechanisms affecting the lesion stability and the risk that re-learning might overshadow the effects of the transplant (Dowd and Dunnett, 2004, 2005a, 2005b).

Here we use a novel variant of the lateralised choice reaction time task to provide a more in-depth analysis of both motor and non-motor deficits and recovery in dopamine-depleted and dopamine cell-rich grafted rats, as well as extending our understanding of the role of dopamine in responding in contralateral space. Specifically, we explore (i) the effects of near complete lesions aimed at the medial forebrain bundle (MFB) on a near/far version of the lateralised choice reaction time task, (ii) whether the lesion-induced deficit is stable over time, and (iii) the effects of restoring striatal dopamine levels by means of grafting dopamine rich neural precursors derived from E14 embryonic VM, on both motor and non-motor parameters.

## Materials and methods

All procedures were performed according to the United Kingdom Animals (Scientific Procedures) Act, 1986 and approved by local ethical review at Cardiff University.

### *Subjects*

A cohort of 32 female Lister Hooded rats (Charles River, UK) started operant training at 12 weeks of age (mean weight: 200–225 g). Rats were housed in standard laboratory cages with 3–4 rats per cage, at a constant temperature of $21 \pm 1$ °C and 50% humidity. The day–night cycle was set to 12:12 h, with the light turned on at 07:00 am. Rats were food restricted to 90% of their free-feeding body weights starting one week prior to operant training, by providing them with weighed amounts of food at the completion of each day's testing. They were allowed ad libitum access to water in the home cages throughout all stages of the experiment.

### *Apparatus*

Operant testing was conducted in standard 9-hole operant chambers (Campden Instruments, Loughborough, UK), which have been described in detail previously (Carli et al., 1983; Dowd and Dunnett, 2004). Briefly, the operant chamber is fitted with a horizontal curved array of 9 response holes which can be illuminated by a green LED and into which a nose poke is detected by break of an infrared beam. A food magazine is fitted in the opposite side of the chamber, to which a pellet dispenser is connected. During operant training and testing, only the centre hole in the array and the two adjacent holes on either the left or the right of the centre hole (depending on the day of testing, see below) were left open, whereas all other holes were covered with well blanks. The 9-hole boxes were controlled by the Cambridge Cognition Control software (Campden Instruments, version 1.23) running on a standard desktop PC using the Windows XP operating system.

### *Training*

Rats were habituated to the operant boxes during a 30 min session in which 30 precision food pellets (45 mg, Sandown Scientific, Hampton, Middlesex, UK) were delivered via the pellet dispenser to the food magazine at the beginning of the session. During the second magazine training session, rats were put into the operant chambers with the house light illuminated. After a short interval the house light was extinguished and the light behind the panel of the magazine was illuminated. Each nose poke into the illuminated magazine resulted in a pellet reward.

Upon conclusion of magazine training, nose poke training commenced. Initially, the centre hole (hole 5) in the array was uncovered and illuminated at the beginning of each trial while all other lights were turned off. A nose poke into the illuminated centre hole resulted in the delivery of a food pellet in the magazine. At the same time, the centre hole light was extinguished and the light in the magazine was illuminated until the food pellet was collected, at which point a new trial commenced.

Once all rats learned to nose poke, the two holes on just one side of the centre hole were uncovered (holes 3 and 4 to the left, or holes 6 and 7 to the right) alternating between the two sides on successive days. Rats were required to respond in the illuminated centre hole (hole 5), which resulted in illumination of one of the lateral holes (holes 3 and 4 on odd days, holes 6 and 7 on even days; see Fig. 1). The lateralised stimulus light remained lit until the rat responded with a nose poke, at which point a food pellet was delivered into the magazine. After achieving >80% correct trials on the basic task, the stimulus duration was gradually reduced from continuous to 200 ms and the required duration of the centre nose poke (hole 5) hold was gradually increased. Once all the rats performed at an asymptotic level on the task, at the longest centre poke duration (400 ms), the rats were trained on the final configuration for a period of three weeks. This consisted of sessions in which the centre hold duration was pseudo-randomly chosen between 4 different hold durations (50 ms, 100 ms, 150 ms, and 200 ms), and the duration of stimulus illumination was always 200 ms. The variable centre holds served to reduce the incidence of anticipated nose withdrawals from the centre hole, thereby enhancing the accuracy of the reaction time measure. The last two weeks of pre-lesion training was recorded as baseline data. A correct response resulted in the delivery of a food pellet into the food magazine, whereas an incorrect response or a premature withdrawal resulted in a "punishment" of a time out period of 5 s where all lights were extinguished (schematic outline in Fig. 1).

The main outcome measures recorded for each session are:

- *Trials usable*, the number of usable trials was defined as those in which the rat responded to the illuminated centre hole for the required delay, initiating the presentation of the stimulus light.
- *Accuracy*, the percentage of correct responses made on each side, for the near and the far hole respectively, divided by the total number of usable trials with the matching stimulus.

Please cite this article as: Heuer, A., et al., Dopamine-rich grafts alleviate deficits in contralateral response space induced by extensive dopamine depletion in rats, Exp. Neurol. (2013), http://dx.doi.org/10.1016/j.expneurol.2013.01.020

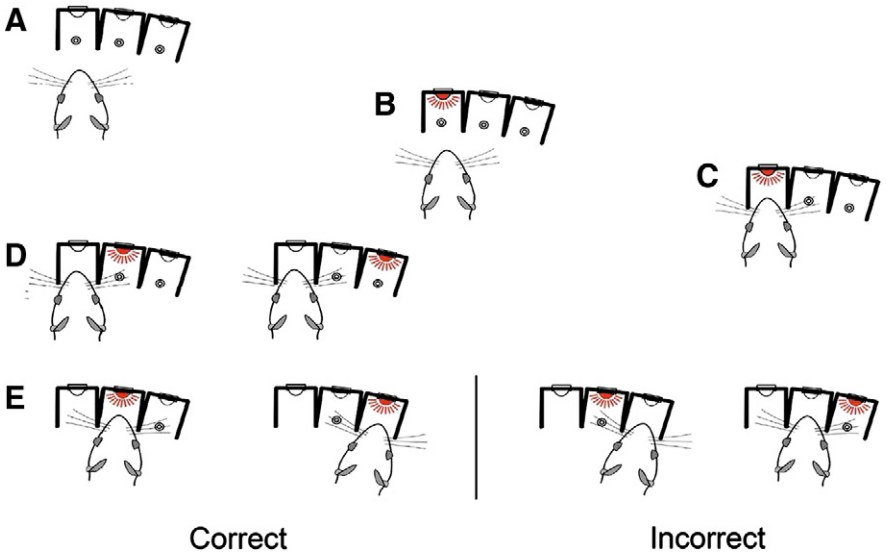


**Fig. 1.** Schematic outline of the choice reaction time task used in the present study (the side is dependent on the day of testing, and on the right side in this example): (A) the animal is set in the operant chamber with only the centre hole and the two adjacent holes being exposed. (B) At the start of each trial, the stimulus light in the centre hole is illuminated. (C) The animal has to perform a sustained nose-poke (50 ms, 100 ms, 150 ms, 200 ms) in the illuminated centre hole. (D) The centre light is switched off and the stimulus light in one of the adjacent holes is simultaneously illuminated. (E) A nose-poke in the illuminated hole (E Correct) marks a correct response which is rewarded with a precision sugar pellet, whereas a nose poke into the non-illuminated hole (E Incorrect) results in a 5 s time-out "punishment" for the animal, during which all lights are switched off.

- *Reaction time*, the mean latency to initiate a response, calculated separately for correct responses and incorrect responses to near and far holes on the left and right sides, from onset of the lateral stimulus light to withdrawal of the nose from the centre hole.
- *Movement time*, the mean latency to execute the choice response, calculated separately for correct responses and incorrect responses to near and far holes on the left and right sides, from the withdrawal of its nose from the centre hole to nose-poking the correct (lit) or incorrect (unlit) hole.

### *Procedure*

After pre-lesion training, rats were rank-ordered on the basis of their accuracy in the task and divided into three matched groups. Rats were given one week of ad libitum access to food prior to surgery. Two groups (each n = 11) received unilateral 6-OHDA injections into the MFB, the remaining rats (n = 10) were left as untreated controls. Three weeks post-surgery, rats were food restricted again and tested for 6 days (3 days per side, alternating) to assess the influence of the lesion on performance in the task. The lesioned rats were rank ordered again to ensure the two groups displayed comparable levels of post-lesion performance. Half of the lesioned rats (Graft; n = 11) received E14 VM grafts, as described below, whereas the remaining rats remained lesion only (Lesion; n = 11). Three months post-transplantation, all rats were food restricted and tested for 50 consecutive days on the operant choice reaction time task (alternating sides each day, starting ipsilateral). After 50 days of testing, several probe trials were conducted to investigate the effects of altering the stimulus duration and changing the configuration of the response locations.

### *Lesion surgery*

Anaesthesia was induced using 5% isoflurane with oxygen as carrier gas. Thereafter, anaesthesia was maintained during surgery by 1–2% isoflurane in a 2:1 $O_2$/NO gaseous mix. The skin over the surgical area was shaved, and the rat was placed in a Kopf model 900 stereotaxic frame with the nose bar set to −4.5 mm. To produce unilateral lesions of the MFB (Torres and Dunnett, 2007), the neurotoxin 6-OHDA (hydrobromide salt, Sigma Chemicals, UK) was injected at stereotaxic coordinates (from bregma): AP −4.0 mm (behind bregma), ML −1.3 mm (right hemisphere) and DV −7.0 mm (below dura). The toxin was infused via a 30-gauge stainless steel cannula connected by fine polyethylene tubing to a 10 μl Hamilton syringe in a micro-pump set to a flow rate of 1 μl/min. In total 3 μl of 6-OHDA was delivered at a concentration of 4 μg/μl (calculated from freebase weight), dissolved in a solution of 0.2 mg/ml ascorbic acid in 0.9% sterile saline. Injections were made over a period of 3 min after which the injection needle was left in place for an additional 3 min to allow for diffusion of the toxin before the injection needle was carefully retracted. After surgery, the wound was cleaned and sutured and the rat was placed in a heated recovery box. Post-operative analgesia was provided by the addition of paracetamol (500 mg dissolved in 0.5 l) to the drinking water for three days post-surgery.

### *Graft surgery*

For transplantation of the primary fetal VM, one pregnant Lister Hooded dam was sacrificed when embryos were of gestational age E14.5 (CRL = 11–12 mm). Cell suspensions were prepared according to a standard protocol (Mayer et al., 1992). In total 15 embryos were dissected and the tissue was pooled in 60 μl of medium resulting in a total yield of $6.5 \times 10^6$ cells with 99.7% viability as assessed by trypan blue exclusion. Cells were re-suspended to a density of 100,000 cells/μl and injected into the denervated striatum at the following coordinates in mm from bregma: (i) AP +1.2, ML −2.2; (ii) AP +0.5, ML −2.9; (iii) AP −0.3, ML −3.6, and (iv) AP −1.1, ML −4.2, at three depth below dura for each of the burr holes (DV −5.5, −5.0, −4.5). A total volume of 4 μl was injected over the four injection sites with the injection needle slowly raised over the three depths over the injection period of 3 min (1 min at each depth). The grafting cannula was left in place for an additional 3 min to allow for diffusion of the suspension before slow retraction. Post-op care was as described above (see Lesion surgery section).

### *Simple behavioural screens*

The success of the lesion surgery and the functional efficacy of the grafts were assessed using the drug-induced rotation test at multiple

Please cite this article as: Heuer, A., et al., Dopamine-rich grafts alleviate deficits in contralateral response space induced by extensive dopamine depletion in rats, Exp. Neurol. (2013), http://dx.doi.org/10.1016/j.expneurol.2013.01.020

time points (2 and 4 weeks post-lesion; 3, 6, and 20 weeks post-transplantation). Rotation was assessed in automated rotometer bowls which were built after the design of Ungerstedt and Arbuthnott (1970). Amphetamine-induced rotation was induced by intraperitoneal injection of 2.5 mg/kg methamphetamine hydrochloride (Sigma Chemicals, UK) dissolved in sterile saline and behaviour was recorded over a period of 90 min. All rotation scores were expressed as an average of ipsilateral rotations minus contralateral rotations (Torres and Dunnett, 2007).

### *Histology*

Following the completion of testing (week 20 post-transplant), rats were deeply anaesthetised with 200 mg/ml sodium pentobarbitone and transcardially perfused with 100 ml of phosphate buffered saline (PBS) pH = 7.4, followed by 250 ml of 4% paraformaldehyde in PBS. The brains were carefully removed from the skull and post-fixed in 4% paraformaldehyde in PBS for 24 h, after which they were placed in 25% sucrose solution in PBS until they sunk. To visualise the extent of the lesion, coronal brain sections were cut on a freezing sledge microtome at a thickness of 40 μm into 0.1 M TRIS buffered saline (pH = 7.4, TBS) and stored at +4 °C prior to staining. Immunohistochemical analysis of tyrosine hydroxlase (TH) was conducted on a 1 in 6 series of free-floating sections, by the streptavidin–biotin reaction using a DAKO kit and with 3,3′-diaminobenzedine as chromogen, as described previously (Torres and Dunnett, 2007).

### *TH-ir cell counts*

For the assessment of lesion extent all TH-immunoreactive (TH-ir) cells in the ipsilateral and contralateral SN and VTA were counted in one section of the ventral midbrain for all rats on a Leica DM/RBE microscope. The medial terminal nucleus of the accessory nucleus of the optic tract was used to define the boundary between the SN and the VTA and to select the respective midbrain section. All cell counts were expressed as percentage of the contralateral side. For the assessment of surviving grafts every TH-ir cell was counted across a 1:6 series of sections on an Olympic C.A.S.T. grid system. 100 TH-ir cells across all grafts were randomly selected and their average diameter was measured. The total number of surviving cells was estimated using Abercrombie's correction procedure (Abercrombie, 1946): $T = F \times A \times M/(D/M)$, where T = total number of cells, F = frequency of sections, A = total cell counts, M = section thickness, and D = average cell diameter.

### *Statistics*

Statistical analysis of the behavioural data was carried out using the GENSTAT v13.1 statistical software package (VSN International Ltd.; Hemel Hempstead, UK). For all analyses a significance level of $\alpha = 0.05$ was used. For post-hoc comparisons, Newman–Keuls' tests were used, as appropriate. The operant behavioural data were analysed using repeated-measures analyses of variance with Group (Control, Lesion, Graft) as the between-subjects factor, and Side (Ipsilateral, Contralateral), Week (Baseline, Lesion, Transplant 1 to 5) and Distance (Near, Far) as within-subject factors. The comparison of mean latencies uses geometric, rather than arithmetic means, for each rat's daily reaction and movement times, to reduce the influence of outlier data points.

## Results

One rat in the control group died over the course of the experiment, unrelated to any treatments. Furthermore, after analysis of rotational behaviour in response to 2.5 mg/kg methamphetamine and after examining the brain sections for the presence of TH-ir cell bodies (see below; Immunohistochemistry for tyrosine-hydroxylase section) we excluded animals that did not have visible surviving grafts and therefore the final group sizes available for analysis were: Control, n = 9; Lesion, n = 11; Graft, n = 5.

### *Amphetamine-induced rotation*

Drug induced rotations per experimental time-point are presented in Fig. 2. There was a significant difference in the number of rotations per group (Group, $F_{2,22} = 63.65$, $p < 0.001$) and this effect was different for the groups between the weeks of testing (Week × Group, $F_{8,88} = 15.71$, $p < 0.001$). The lesion group rotated at a rate that was significantly higher than the control group when challenged with amphetamine in all test sessions ($p < 0.001$). The graft group had rotation scores as high as the lesion group (n.s.) during the first two weeks of assessment (i.e., during the two post-lesion sessions). Rotation was reduced following transplantation, such that by 6 weeks post-grafting the performance of the grafted group was similar to the control group (n.s.) and significantly lower than rotations of the lesion group ($p < 0.001$). There was no difference in rotation scores for any of the groups between the last two rotation sessions.

### *Immunohistochemistry for tyrosine-hydroxylase*

Representative examples of coronal sections stained for TH-ir for each of the three groups are presented in Fig. 3. Injection of 6-OHDA into the MFB caused degeneration of TH-ir terminals in the ipsilateral striatum (Fig. 3B) and degeneration of the dopaminergic cells in the SNc and part of the VTA on the ipsilateral side of injection (Fig. 3E). The extent of the lesion was assessed by visual confirmation of denervation of TH-ir fibres and terminals in the striatum and quantified by TH-ir cell counts in the host VM. Cell counts at the level of the accessory optic nerve for the SNc and the VTA (Table 1) were significantly reduced in rats that underwent the lesion surgery, relative to controls (Group, $F_{2,27} = 533.70$, $p < 0.001$) and no difference between the Lesion only and Graft group was evident (n.s.). The number of TH-ir cells in the other ventral midbrain dopaminergic nucleus, the VTA, was also affected by the lesion (Table 1) but to a lesser extent than the SN (Table 1, Group, $F_{2,27} = 9.94$, $p < 0.001$). No differences in VTA cell counts were found between the two lesion groups (both, n.s.), which in turn both differed significantly in their cell numbers from control (Lesion, $p < 0.01$; Graft, $p < 0.05$).

Dopamine-rich grafts re-innervated the denervated striatum (Fig. 3C) and contained cells staining positive for TH (TH-ir cell count: 2805 ± 356), mostly in the periphery of the graft (Fig. 3F). However, analysis of the tissue revealed viable grafts in only five of the grafted rats, whereas six animals had no viable grafts and only needle tracts were visible. These latter cases were excluded from

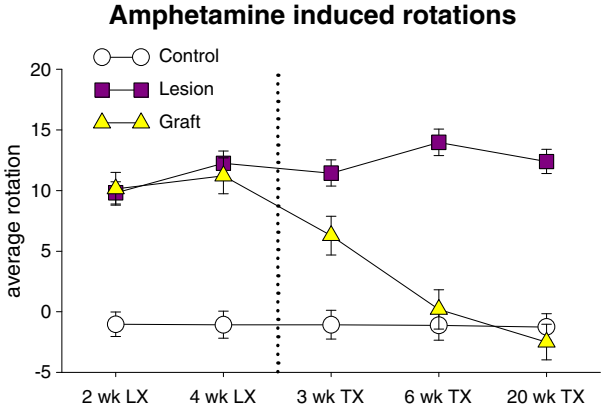


**Fig. 2.** Drug-induced rotations recorded over 90 min in response to 2.5 mg/kg methamphetamine. LX = post-lesion; TX = post-transplantation. Here only animals that had viable grafts were included in the analysis.

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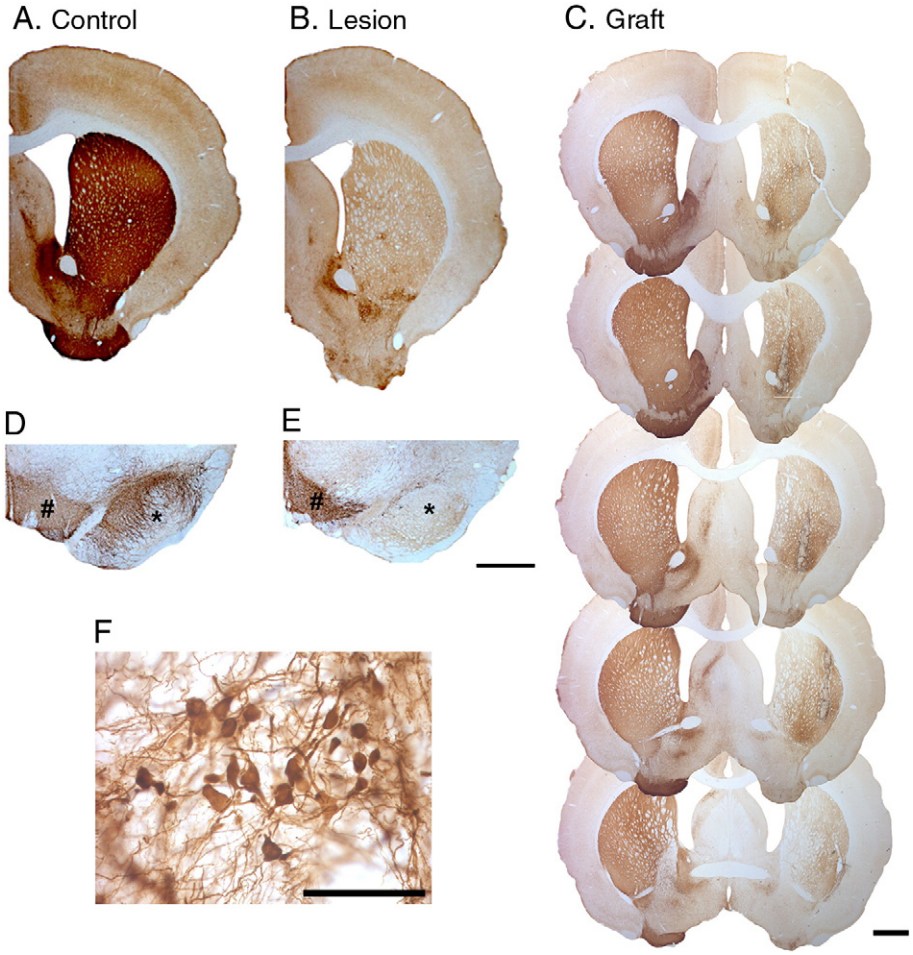


**Fig. 3.** Photomicrographs of representative brain sections of animals of the control group (A, D), the lesion group (B, E) and the graft group (C, F) stained for TH. The hash symbol marks the location of the VTA whereas the asterisk marks the location of the SN. Note the lack of TH-positive fibres in the lesioned striatum (B) and degeneration of TH-positive neurons in the SN (E) of the lesion group. The graft is clearly visibly in the dorsolateral striatum, with graft-derived fibre reinnervation throughout the lateral half to two thirds of the striatum (C) and high-power magnification shows donor derived dopaminergic neurons in the periphery of the graft (F). Scale bar = 1000 μm for A, B, D, and E; 1000 μm for C and 100 μm for F.

the behavioural analysis. As all of the non-surviving grafts were implanted by one experimenter we assume technical factors with the grafting syringe as reason for the low viability of the transplants.

*Results of operant analysis of behaviour*

*Trials usable*

A trial was considered 'usable' when the required hold duration in the centre hole was completed successfully, regardless of whether the subsequent response was correct or not. The mean number of usable trials is shown in Fig. 4A for each group. Data are collated into blocks of 5 days, with the exception of the post-lesion assessment, which consisted of 3 days on each side. There were significant differences in the numbers of usable trials between the three groups per week of testing (Week × Group, $F_{12,132} = 18.92$, $p < 0.001$) but not between the sides of testing (Sides, $F_{1,22} = 1.29$, n.s.). All rats produced a high number of usable trials during pre-lesion training. After the lesion, control rats still produced a high number of usable trials (69.94 ± 1.92), whereas rats in the two lesion groups produced on average less than half the number of usable trials (Lesion: 29.28 ± 1.22; Graft: 32.4 ± 2.58). Interestingly, after transplantation, grafted rats produced more usable trials than lesioned rats, although this did not reach the level seen in the control rats ($t_{22} = 4.88$, $p < 0.01$).

**Table 1**
Dopaminergic cell counts in the ventral mesencephalon for each experimental group.

| Test | Control | Lesion | Graft |
|---|---|---|---|
| SN (%) | 99.62 ± 4.22 | 6.00 ± 1.58 | 2.78 ± 0.94 |
| VTA (%) | 98.12 ± 4.46 | 69.18 ± 8.45 | 57.22 ± 4.52 |

Data represent mean and standard error of measurement.

*Accuracy*

Accuracy was defined as the number of correct trials divided by the number of usable trials, expressed as a percentage (Figs. 4B, C). During pre-lesion acquisition, all rats performed with high accuracy and there was no difference between the groups (Group, $F_{2,22} = 1.39$, n.s.) nor the side of testing (Side, $F_{1,22} = 0.33$, n.s.). Accuracy was higher when responses were directed towards the nearer of the two response locations (Distance, $F_{2,22} = 34.51$, $p < 0.001$) and this difference in response accuracy was not different between the groups (Distance × Group, $F_{2,22} = 2.11$, n.s.).

After receiving unilateral nigrostriatal lesions, the lesioned rats displayed a marked reduction in accuracy which was most affected when responses had to be directed to the far contralateral response location (Distance × Side × Group, $F_{2,22} = 9.73$, $p < 0.001$). Restricting the analysis to the side contralateral to the lesion revealed a reduction in accuracy in the two lesion groups (Group, $F_{2,22} = 42.54$, $p < 0.001$) relative to the control group. Although ipsilateral accuracy was slightly reduced in the lesion group, this effect failed to reach statistical significance (Fig. 4C; $F_{2,22} = 2.81$, n.s.).

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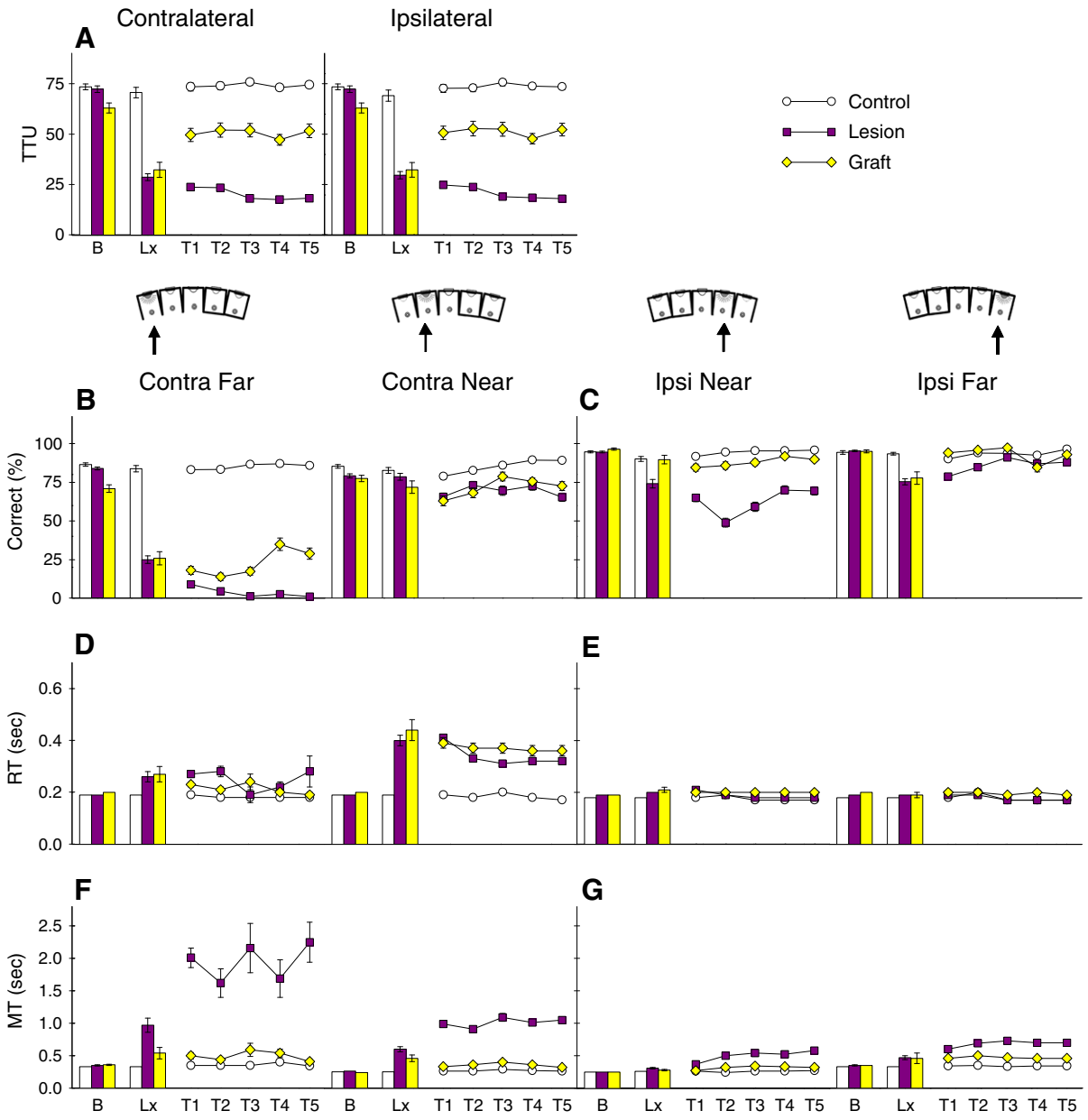


**Fig. 4.** Main outcome measures on the operant choice reaction time task plotted by week of testing for contralateral and ipsilateral days of testing. Base (Baseline) = 5 days; LX (Lesion) = 3 days; TX (Transplant) = 5 days. (A) Total Trials Usable (TTU). (B, C) Percent response accuracy. (D, E) Reaction time (RT) on correct trials. (F, G) Movement time on correct trials.

After engrafting one of the lesion groups, testing continued for 25 days per side and data were collated into 5 day blocks. Analysis of the test sessions post-transplantation revealed an interaction between the groups, the side of testing and the response location for the accuracy measure (Distance × Sides × Group, $F_{2,22} = 17.77$, $p < 0.001$). Comparing rats' responses on the contralateral side only demonstrated a significant interaction between the week of testing, group and response location (Week × Distance × Group, $F_{8,88} = 3.15$, $p < 0.01$). Whereas the control group responded highly accurately to the far contralateral response location during all days of post-graft testing, the lesion group was significantly impaired in responding accurately to this far hole location, and the graft group improved marginally over the 5 weeks of testing. There was no significant group difference in accuracy on the contralateral near hole (Group, $F_{2,22} = 2.04$, n.s.) and response accuracy on the contralateral near hole did not change over the course of testing (Week X Group, $F_{8,88} = 1.15$, n.s.; analysis restricted to the near response location). When rats were tested on the side ipsilateral to the lesion there was a significant difference between the three groups over the 25 days of post-transplantation testing (Group, $F_{2,22} = 14.41$, $p < 0.001$). This effect did not change as function of week (Week × Group, $F_{8,88} = 1.93$, n.s.). The effect of response location was also not different between the three groups on the ipsilateral side (Distance × Group, $F_{2,22} = 2.13$, n.s.). Interestingly, the post-graft ipsilateral accuracy was highest in the control group and lowest in the lesion only group, with both the Control and Graft groups performing significantly better than the rats with lesion alone (Newman–Keuls, $p < 0.001$ and $p < 0.05$, respectively).

*Reaction time*

Reaction time on correct trials was defined as the latency to withdraw from the sustained centre nose poke upon illumination of a lateralised stimulus light (see Figs. 4D, E). During pre-lesion acquisition, there was no interaction between the groups, side of testing and response location for the reaction times (Distance × Sides × Group,

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$F_{2,22} = 0.64$, n.s.). Post-lesion, the reaction times increased for rats of the lesion groups (Group, $F_{2,22} = 12.94$, $p<0.001$) and differed according to response location and side of testing (Distance × Side × Group, $F_{2,22} = 4.51$, $p<0.05$). Whereas reaction times of the control group were similar for near and far hole responses, they were increased in the two lesion groups with longer reaction times for near hole responses. The increase in reaction time was more prominent when lesioned rats were tested on the side contralateral to the lesion. During the 5 weeks of post-graft testing, reaction times did not change for each of the three groups (Week × Distance × Group, $F_{8,88} = 0.47$, n.s.). Restricted analysis of reaction time performance on the contralateral side alone revealed a group difference (Group, $F_{2,22} = 9.25$, $p<0.01$) and that demonstrated that reaction time was longer when the stimulus was presented in the near hole (Distance, $F_{1,22} = 11.36$, $p<0.001$).

*Movement time*

Movement time was defined as the time taken to respond to the stimulus and was measured starting from the withdrawal of the nose from the centre hole to the entrance of the nose into the correct response location. As can be seen in Figs. 4F and G, movement times were similar for the three groups during pre-lesion training (Group, $F_{2,22} = 1.24$, n.s.) and no differences were evident between the sides of testing (Sides, $F_{1,22} = 0.28$, n.s.). As expected, response latencies were longer when executed to the far response hole than to the near response hole (Distance, $F_{1,22} = 244.95$, $p<0.001$), and this effect was similar for all three groups (Distance × Group, $F_{2,22} = 0.89$, n.s.).

Post-lesion, an effect of group was revealed when rats were tested on the side ipsilateral to the lesion (Group, $F_{2,22} = 8.45$, $p<0.01$) with the two lesion groups responding slower on average than the control group. Movement times remained slower for responses to the far response location (Distance, $F_{1,22} = 26.51$, $p<0.001$) but this effect was similar for all three groups (Distance × Group, $F_{2,22} = 1.44$, n.s.). As can be seen in Fig. 4 movement times for far contralateral responses were much slower for the lesion only groups. Response execution on the side contralateral to the lesion was affected similarly to ipsilateral movement times, although the effect was larger. There was also a difference in movement times between the three groups (Group, $F_{2,22} = 9.61$, $p<0.001$), with the lesion group responding much slower compared to the other two groups. Movement times were faster for responses made to the near contralateral location (Distance, $F_{1,22} = 6.12$, $p<0.05$) and for all three groups responses to the farther response location took longer to execute (Distance × Group, $F_{2,22} = 1.68$, n.s.).

After transplantation, the graft group differed in movement times executed to the ipsilateral and contralateral sides (Side, $F_{1,22} = 16.50$, $p<0.001$). On the ipsilateral side, the post-graft performance was similar to the post-lesion performance, with a difference evident between the three groups (Group, $F_{2,22} = 16.47$, $p<0.001$) and an increased time to respond to the far ipsilateral location (Distance, $F_{1,22} = 17.77$, $p<0.001$), an effect that did not differ between the groups (Distance × Group, $F_{2,22} = 1.97$, n.s.). Across both sides and response locations, movement times did not change as a function of week of post-graft testing (Week, $F_{4,88} = 1.99$, n.s.). This effect was not different between the groups (Week × Group, $F_{8,88} = 0.92$, n.s.). On contralateral days of post-graft testing, rats displayed a similar pattern of movement times towards near and far response holes as on ipsilateral testing days, although the differences were more pronounced on the contralateral side. There were differences in movement times between the groups (Group, $F_{2,22} = 16.88$, $p<0.001$) with the control group responding faster than the lesion and graft groups. Whereas the lesion only group had significantly slower movement times than the control group ($t_{22} = 8.26$, $p<0.01$) and the graft group ($t_{22} = 6.33$, $p<0.01$) there was no significant difference between the graft and the control groups ($t_{22} = 1.93$, n.s.). There was no change in movement times over the 5 blocks of post-graft testing (Week, $F_{4,88} = 1.27$, n.s.). Interestingly only the lesion group responded with increased movement time latencies towards both the near and the far contralateral and ipsilateral response locations, whereas the graft group responded with speeds similar to those of the controls.

*Probe trials*

After completion of the 50 consecutive days of post-transplantation testing, two probe trials were conducted to assess the performance deficit in further detail, by manipulating task demand and altering the response requirements. Firstly, the effect of manipulating the duration of the stimulus light was investigated and, secondly, the response locations were shifted to new, more distal, locations. Testing of each probe manipulation occurred over 10 consecutive days (5 days per side) and always started with testing on the side ipsilateral to the lesion. One rat of the lesion group died in the course of probe testing (under circumstance unrelated to any procedure) and was therefore excluded from further analyses. When analysing probe trials response accuracy was expressed as near hole bias ($\text{Bias} = \text{Accuracy}_{near}/(\text{Accuracy}_{near} + \text{Accuracy}_{far}) \times 100$).

*Manipulation of the stimulus duration*

In order to assess the impact of stimulus duration on performance, the duration of the required sustained nose-poke in the centre hole was reduced such that rats were only required to sustain the hold for 50 or 100 ms. The stimulus light, which indicated the correct response location, was then pseudo-randomly selected to be either 400, 200, 100, or 50 ms in length. All other parameters were kept constant. Interestingly, all groups performed similarly with regard to the number of usable trials executed, and the reaction and movement time latencies, in spite of the manipulation of the stimulus duration (data not shown). The manipulation had a small effect on the near hole bias, which was different between the three groups (Fig. 5A, Stimulus Length × Group, $F_{6,61} = 6.81$, $p<0.001$). The lesion group displayed almost exclusive responding towards the near response location, irrespective of the stimulus length, compared to controls (all $t_{22} > 7.59$, $p<0.01$) and the grafted group (all $t_{22} > 4.24$, $p<0.05$). Only at the very shortest stimulus duration failed the difference in response accuracy between animals of the control group and the engrafted group to reach significance ($t_{22} = 3.35$, n.s.). As can be seen in Fig. 5A, only the control group was affected by the stimulus duration, with shorter stimulus lengths leading to bias responses towards the nearer response location.

*The effects of shifting the response location*

Next, the effect of manipulating the response location was assessed, such that the previous far hole became the nearest and a new hole was unblocked to become the new far location. The unused response hole next to the centre hole was, as all other non-utilised holes, covered and not accessible to the rat. Similar to the post-transplantation test period, rats that underwent lesion surgery displayed a greater near hole bias than the control group (Group, $F_{2,21} = 26.40$, $p<0.001$, Fig. 5B). The graft group again demonstrated a moderate, but significant, improvement in performance when compared to the lesion group (Lesion, Graft; both, $p<0.001$), but still directed significantly more responses to the near response location than control rats did (Lesion, Graft; both, $p<0.001$). Interestingly, despite demonstrating severe impairments in responding to the far hole during the initial post-lesion and post-graft testing, rats in the lesion only and graft groups were unimpaired in responding to that same response location when, relatively-speaking, it was shifted to being the 'nearer' response hole.

## Discussion

The present experiment assessed the effects of near-complete unilateral striatal DA depletion on an operant choice reaction time task in

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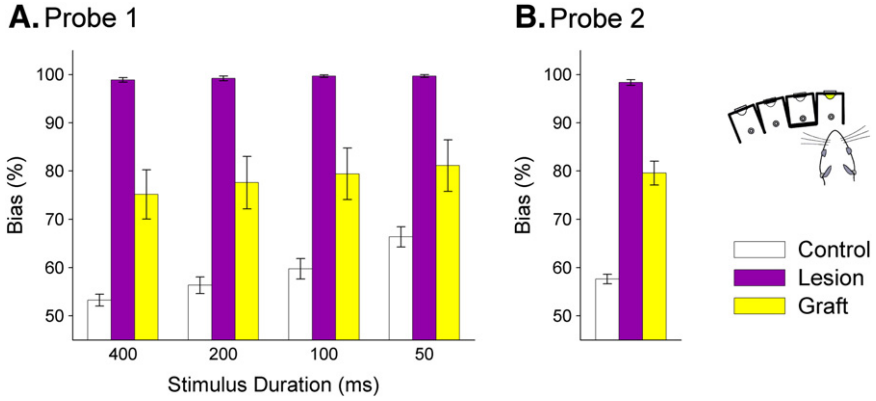


**Fig. 5.** Probe trials that were conducted after post-graft data had been collected. (A) Probe trial 1 (near hole bias %): the effect of stimulus manipulation on the deficit/recovery for responses to the contralateral side. Use of only two hold durations (very short i.e. 50 and 100 ms) and randomly presenting the stimulus light for 50, 100, 200 or 400 ms. (B) Probe trail 2 (near hole bias %): the effect of moving the response hole (i.e. previously far hole becomes the nearer hole of the two-hole choice) for contralateral days of testing. Animals tested for 5 days/side, starting ipsilateral to the lesion (data not shown).

rats and the effects of dopamine replacement therapy on multiple aspects of behaviour. The aim of the present experiment was threefold. Firstly, we assessed and characterised the response profile of near-complete DA depletion on the present version of this task as only partial lesion models have been utilised previously. Secondly, we assessed the lesion-induced deficit at two time points, (i) acute test, shortly (3 days/side) after the lesion and (ii) chronic test, over 50 consecutive days (25/side) beginning 4 months after the lesion. Thirdly, we assessed the ability of transplanting DA-rich tissue into the denervated striatum to alleviate lesion-induced deficits.

The main findings of the present study were that, firstly, the lesion caused a marked reduction in response accuracy when the stimulus light was presented in the far contralateral location, which was accompanied by increases in reaction and movement times on contralateral days of testing. Secondly, the lesion-induced deficit was stable over the weeks of testing, and thirdly, the effects of the graft were small, but highly significant, with an increase in usable trials and an improvement in far contralateral response accuracy, which was accompanied by a marked reduction in contralateral movement time. All effects observed were stable over the 50 days of consecutive testing. All three main findings of the present study are novel and will contribute to the progression of cell based therapies to the clinic. This is the first time that the more complete bundle lesion model has been applied in combination with dopamine-rich grafts on the variant of the lateralised choice reaction time task introduced by Brown and Robbins (1989a). Furthermore, the task utilised here provides a more in-depth analysis of behaviour than did previous versions of the lateralised choice reaction time task. Next to reporting ipsilateral deficits that have not been reported in the literature after dopamine depletion in rats in choice reaction time tasks, the present experiment showed that grafts not only improve the animals deficit on the contralateral side but also on the ipsilateral side as well when each is probed independently.

*Lesion-induced deficits*

The impact of the near-complete unilateral lesions on performance further supports previous findings, that unilateral lesioned rats display longer reaction times towards contralateral stimuli and increases in movement times, when a response is required towards the side contralateral to the lesion (Brasted et al., 1998; Carli et al., 1985; Döbrössy and Dunnett, 1997; Dowd and Dunnett, 2005a, 2005b). The impact of terminal striatal lesions, which result in a more modest depletion of DA, has been reported on a conceptually similar experiment and here the authors demonstrated an impairment only on responses directed in space contralateral to the lesion (Brown and Robbins, 1989a, 1989b). The present experiment revealed a similar pattern of impairment in rats with MFB lesions, in which significantly more DA depletion is evident in both the dorsal and ventral striatal regions. The fact that shifting the response locations further away from the centre hole enabled lesioned rats to execute responses to a high degree of accuracy in a location that had previously been the most distal, and consequently the most impaired, suggests that the ability to execute responses in contralateral space remains intact, while spatial coding of the response or the representation of egocentric space is impaired (Brown and Robbins, 1989a, 1989b). Indeed, in the present study, lesioned rats are able to execute responses to one of the contralateral stimuli with a high degree of accuracy and reasonable movement times, indicating a sparing of sensory processing facilities and no primary motor impairment since the animals are still capable of responding to the neglected contralateral location when the response hole was shifted. Furthermore, the deficits in accuracy and motor function were stable over the time course of testing and did not show signs of spontaneous behavioural recovery. This is important for long-term testing of therapeutic interventions in animal models of disease, since spontaneous recovery and compensatory mechanisms can limit the use of the intra-striatal method of DA depletion (Dowd and Dunnett, 2004). Interestingly ipsilateral effects of MFB 6-OHDA lesion have not been reported previously on response accuracy whereas an increase in movement time was shown before (Dowd and Dunnett, 2004, 2005a, 2005b). During the long term post-graft assessment both experimental groups and the lesion group especially displayed ipsilateral deficits on near hole accuracy and movement time latencies towards both ipsilateral response locations. The main difference to the work of Dowd and Dunnett (2004) is the task utilised. Whereas in previous studies the response options were located to either side of the animals' head, i.e. responses had to be made either ipsilateral or contralateral within a session, in the present study these were assessed on alternating days. Ipsilateral deficits might not have been detected in this setting because animals bias their responses towards the ipsilateral hole. In a follow-up study, we have shown a small ipsilateral deficit in a conceptually similar task with the presence of an ipsilateral deficit depending partly on hole configurations and partly on task demands (Heuer and Dunnett, 2013). The study of Brown and Robbins only tested animals on the same side and placed the lesion either ipsilateral or contralateral to the side of testing (Brown and Robbins, 1989a, 1989b). There are two explanations for the differences between the two studies, firstly the lesion was less pronounced as partial lesions largely spare cells in the VTA and therefore dopamine innervation of the ventral striatum/nucleus accumbens, and secondly continuous testing on one side might have masked ipsilateral effects as the ipsilateral response is the default response by the lesioned animal.

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DA depletion disrupts a spatial system in which responses are coded in absolute contralateral space and it was shown that blocking the nearer of two contralaterally located holes allowed improved direction of responses into the previously more distal location (Brown and Robbins, 1989b). Increasing the duration of the stimulus light during the probe trials was intended to decrease task demand by eliminating the reliance on working memory function and negating the need to code spatially the response location from the centralised position, but, interestingly, manipulation of the stimulus duration (Probe 2) had no effect on the performance of the lesion group. Moreover, the lesioned animals always directed their responses to the nearer of the two contralateral response options, irrespective of the absolute spatial location of these (compare standard configuration to Probe 1), which provides further support for the theory that responses are misdirected in contralateral space, rather than an inability to detect (primary sensory impairment) or make responses to (primary motor impairment) the contralateral stimulus. When the stimulus duration is extremely short (i.e. 50 ms) even animals of the untreated control group bias more of their responses towards the near contralateral hole compared to long stimulus duration (i.e. 400 ms).

*Response bias as a failure of space representation*

It has been proposed that the accuracy deficit is due to a distortion in space representation. Response space and representational neglect can be ascribed to either of three possible frames of reference, (i) egocentric or viewer centred neglect, (ii) allocentric or environmentally centred neglect, or (iii) object centred neglect, irrespective of the objects location in space (Kerkhoff, 2001). In the absence of an ipsilateral impairment, responses in far contralateral space were changed after the lesion, supporting the view of en egocentric deficit in absolute contralateral space (Brasted et al., 1997, 1999a, 1999b, 2000; Brown and Robbins, 1989a, 1989b). When faced with only one response option on the side contralateral to the lesion, i.e. in a simple reaction time task setting, lesioned rats were able to respond correctly to both, near and far, spatial locations, supporting the argument that responses are misdirected rather than an inability to locate the stimulus in space (Brown and Robbins, 1989a, 1989b). The pattern of responding in the present study further supports theories of failure to direct responses in the presence of competing response locations in absolute egocentric space where sensorimotor deficits are secondary (Brasted et al., 1997; Brown and Robbins, 1989a, 1989b). Interestingly grafting rats with DAergic cells restored the animals' ability to respond towards the far contralateral stimulus location. Although this ability to direct responses into far contralateral space is not recovered in the true sense that grafted animals display equal performance to those of the control group, but given that animals of the lesion group did bias their responses almost completely towards the contralateral near hole, a response accuracy of $>25\%$ of far hole is a remarkable achievement with the type of graft used.

*Effects of grafts*

The grafts alleviated many of the deficits induced by the lesion, including increasing the number of trials attempted, improving the response accuracy on contralateral trials and reducing movement times. Indeed, a previous study (Dowd and Dunnett, 2004) demonstrated the efficacy of cell replacement strategies using dopaminergic donor tissue in the unilateral 6-OHDA lesion model. There, the graft group displayed a modest but significant increase in contralateral response accuracy and a substantial increase in the number of trials attempted (Dowd and Dunnett, 2004), which is in accordance with the improvements produced by cell replacement therapy in the present paradigm. In both studies the grafts failed to provide complete recovery of function but improved the rats' performance compared to those of the lesion groups. Furthermore, in the present study, the graft effect was stable over time and grafted rats performed significantly better than those of the lesion group. In previous reports the effect of the graft on response accuracy could only be seen for two weeks and was not different from the lesion only group from day 14 onwards on this parameter (Dowd and Dunnett, 2004). In the present experiment, using more extensive lesions, we can report that the effect of the graft is stable even after 50 days of consecutive testing (25 days/side). Interestingly, the graft had an effect on ipsilateral days of testing when performance is compared to the lesion only group. Whereas response accuracy was also reduced in lesioned animals on the ipsilateral side, and movement times were increased, the grafted animals performed similar to unlesioned controls on both parameters. Although ipsilateral effects of grafts have not, to our knowledge, been assessed in the 6-OHDA lesion model in choice reaction time tasks, it has been shown that unilateral grafts in patients can alleviate ipsilateral symptoms, such as reducing bilateral arm rigidity and improving movement speed, especially in flexion movements (Lindvall et al., 1990). Although recovery was higher on contralateral parameters, the ipsilateral facilitation of movement after engraftment of dopamine rich mesencephalic tissue is remarkable (Lindvall et al., 1990). Whereas small ipsilateral improvements in movement time latencies have been reported after transplantation into Parkinson's disease and Huntington's disease models (Brasted et al., 1999a, 1999b; Dowd and Dunnett, 2004), we believe this to be the first study to report an improvement of ipsilateral response accuracy in the 6-OHDA lesion model in rats using primary fetal mesencephalic donor tissue.

Here we show that the largest effect the graft had on contralateral performance was in the reduction in contralateral movement times; on this parameter, lesioned rats eventually resembled control animals. Indeed, it has been shown previously that re-introduction of tonic striatal dopamine facilitates movement into contralateral space (Dowd and Dunnett, 2004). Importantly, whereas tonic dopamine is sufficient for movement initiation, the phasic reward signalling is necessary for reward prediction and learning (Montague et al., 1996; Schultz, 2000; Schultz et al., 1997). Whereas Dowd and Dunnett (2007) speculate that the maintenance of the contralateral response is dependent on phasic reward signalling (Dowd and Dunnett, 2007), we demonstrate that the grafts can restore, at least partly, the conditioned contralateral response, as manifested by increased number of trials usable and increased choice response accuracy. Dopamine depletion in the entire striatum – and especially the large depletion of the ventral striatum and nucleus accumbens – has been shown previously to reduce the number of trials attempted (Cousins and Salamone, 1996; Cousins et al., 1993; Darbaky et al., 2003; Dowd and Dunnett, 2005a, 2005b), whereas striatal dopamine depletion does lead to impairments in reaction and movement times, with accumbens lesions having no (additional) effect (Amalric and Koob, 1987; Carli et al., 1989).

The behavioural paradigm utilised in the present study has allowed us to characterise further the role of striatal dopamine in motor and non-motor functions. The efficacy of the cell replacement therapy leads us to hypothesise that this dopaminergic intervention not only improves global motor function, but also specifically reinstates the abilities to encode spatially and to direct attempted responses in egocentric space.

*Limitations of dopaminergic grafts*

The functional efficacy of cell transplantation to combat neurodegeneration is dependent on several factors and not every tissue/cell type allows complete restoration back to control levels. Whereas engraftment of the developing striatum may result in recovery to almost control levels in rat models of Huntington's disease (Brasted et al., 1999a, 1999b, 2000; Döbrössy and Dunnett, 1998; Dunnett, 1995), such a recovery has often not been achieved after replacement

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of dopaminergic cells, even after long term post-graft testing. There may be several reasons for the limitations of dopaminergic cell replacement therapies. Firstly, the site of tissue transplantation is sub-optimal as by transplantation into an ectopic location the cells are deprived of their natural input signalling from the SN. Behavioural effects may therefore be due to a tonic elevation of striatal dopamine, though the phasic dopamine input necessary for reward signalling, normal learning and performance has not been reconstructed (Montague et al., 1996; Schultz, 2000; Schultz et al., 1997). Secondly, the graft only partially restores DA in the transplantation site whereas the depletion caused by the lesion encompasses the striatum, nucleus accumbens, the prefrontal cortex and the olfactory tubercle (Dowd and Dunnett, 2004). Olfaction is the primary sense for rodents and this is one of the main reasons while exploration of the response holes as in the present study leads to faster training than to press a lever as a manipulandum in the Skinner box. Other affected structures such as the nucleus accumbens and the prefrontal cortex have been implicated in general reward signalling, addiction, impulsivity, and motivation/activity levels (Alderson et al., 2001; Bowman and Brown, 1998; Cousins et al., 1993; Nicola, 2007; Salamone et al., 1994; Sesia et al., 2010) and attention and decision making (Birrell and Brown, 2000; Chudasama et al., 2003; Dunnett et al., 2005; Eagle et al., 2008; Joel et al., 1997), respectively.

The extensiveness of the MFB lesion is a major confounding factor that is important to consider when interpreting the results of both post lesion and post graft performance. The main reason for utilizing the near complete lesion model is practical insofar as terminal lesioned rats are prone to spontaneous recovery which renders them useless for the assessment of long term testing that is necessary when investigating the behavioural effects of cell replacement therapies as presented in the present manuscript (Dowd and Dunnett, 2004).

As stated above, the reintroduction of dopamine in the striatum does not directly affect all of these depleted areas. Although, graft location, number of surviving TH-ir cells, reinnervation of TH-ir fibres, does allow for a greater recovery than with suboptimal parameters, some functions have never been ameliorated by dopaminergic grafts whereas striatal (whole ganglionic eminence) grafts were able to produce such a (complete) recovery in excitotoxic lesion models of Huntington's disease (Brasted et al., 1999a, 1999b; Mayer et al., 1992; Montoya et al., 1990).

Although the graft had only small effects in the present study, the improvement compared to lesion only animals is still remarkable. Rats that received grafts not only attempted more trials during the testing session but also markedly decreased the time it took to move towards a stimulus and increased the accuracy of the response. This improvement was stable for 50 days of testing and therefore provides further evidence that cell replacement therapy can provide long lasting effects.

### *Conclusion*

The choice reaction time task in the near-far hole configuration has been shown to be sensitive to near complete depletion of striatal dopamine. Impairments in contralateral performance were highly stable over time and therefore provided an excellent model to investigate the effects of potential interventions, such as cell replacement therapy. Dopamine depletion impaired the number of trials initiated, increased the movement times bilaterally and reduced the response accuracy to contralateral far stimuli, as well as increasing contralateral reaction times. The grafts were able to ameliorate the deficit on many task parameters. Importantly, this demonstrates that replacement of tonic dopamine can facilitate both motor and non-motor performance long-term, and there is a specific impact of tonic dopamine release on the spatial coding of responses in egocentrically-defined space. Furthermore, the results presented here will form the baseline to which alternative cell sources have to be measured. Whereas assessment of simple motor asymmetries such as drug induced rotations provides good indications of lesion extent or cell survival, they are further away from modelling graft-induced improvements than the effects reported here, namely graft-induced amelioration on a task that assesses goal directed behaviour, motivation, and attention.

## Conflict of interests

The authors declare no conflict of interests.

## Acknowledgments

The research leading to these results has received funding from the European Community's Seventh Framework Programme, NeuroStemCell (grant agreement nr. 222943) and the Medical Research Council.

Contribution: A.H. conducted the behavioural part of the experiment. A.H. performed lesion surgery. E.M.T. dissected the embryos and prepared the cell suspensions. A.H. & C.M.K. performed graft surgery. Special thanks to Jane Heath for excellent histological advice. All authors contributed to the final version of the manuscript. A.H., MJL, & SBD designed the experiment.

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Please cite this article as: Heuer, A., et al., Dopamine-rich grafts alleviate deficits in contralateral response space induced by extensive dopamine depletion in rats, Exp. Neurol. (2013), http://dx.doi.org/10.1016/j.expneurol.2013.01.020

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Please cite this article as: Heuer, A., et al., Dopamine-rich grafts alleviate deficits in contralateral response space induced by extensive dopamine depletion in rats, Exp. Neurol. (2013), http://dx.doi.org/10.1016/j.expneurol.2013.01.020

# Chapter 3.3

## Experiment 3: Characterisation of spatial neglect induced by 6-OHDA lesions on a choice reaction time task in rats

The rationale for this experiment resulted out of the findings of experiment number 2 (Chapter 3.2). In the previous study we investigated the effects of near-complete unilateral 6-OHDA-induced DA depletion on a version of the lateralised choice reaction time task in rats. In the adaptation of the task rats were tested on alternating days (contralateral or ipsilateral to the lesion) to respond towards either a proximal or a distal response location. One of the most striking results was the long term stability of the lesion, i.e. even with extensive post lesion training of 50 days (25 days/side, alternating), lesioned rats biased all their responses towards the proximal response location when tested on the contralateral side to the lesion.

Hence, we aimed to further explore the nature of this deficit. We speculated that this could be due either to a true misrepresentation of response space as argued by previous authors or to a change in the animals' response strategy. Indeed in the current task animals that bias all of their responses towards one of the response locations would give a correct response in 50% of all trials. We therefore thought that this might be a cost-benefit decision of the rat so as to put in the effort for attention and memory to get every response correct or to transform the task into a simple reaction time task which is rewarded 50% of all times.

Our primary aim was to 'encourage' rats to respond towards the distal response location and to prevent them from being rewarded for biasing their responses towards the most proximal response location. Therefore we included an error-correction procedure into the task where in case of an erroneous response the same trial is offered again to the rat until a correct response has been made.

The experiment conducted in the present paper as well as analysis of the data, histology and preparation of the manuscript was done by the author of this thesis. Professor S.B. Dunnett was involved in planning of the experiment and gave help and advice throughout as well as in the writing of the manuscript.

Behavioural Brain Research 237 (2013) 215–222

Contents lists available at SciVerse ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

# Characterisation of spatial neglect induced by unilateral 6-OHDA lesions on a choice reaction time task in rats

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## HIGHLIGHTS

- Spatial contralateral neglect is not alleviated by reducing task demand.
- Neglect stable even when animals are "encouraged" to respond to neglected location.
- Neglect is pronounced in a choice, but not simple reaction time task setting.

## ARTICLE INFO

*Article history:*
Received 15 August 2012
Received in revised form
16 September 2012
Accepted 20 September 2012
Available online 27 September 2012

*Keywords:*
Operant
Parkinson's disease
Choice reaction time
9 Hole box
Egocentric
Striatum

## ABSTRACT

Unilateral dopamine depletion and excitotoxic lesions of the striatum have been shown to induce a contralateral neglect when rats have to respond in a choice reaction time setting. Whereas, in a lateralised setting when response options are to either side of the animal's head all contralateral responding is impaired, testing animals only on one side of the head per day but with a near and far response option, rats are able to correctly respond to contralateral stimuli, but rather bias their responses towards the near hole. Here, we further investigated the nature of the contralateral neglect in egocentric space coding in more detail. Firstly, we tested the effects of near-complete unilateral dopamine depletion on this type of task. Secondly, previous observations suggested that lesioned rats shifted their response strategy which resulted in a response bias towards the most proximal location in contralateral space. In order to "encourage" dopamine depleted rats to respond to the neglected response location we implemented an error correction procedure to the task. Near-complete unilateral dopamine depletion, via 6-hydroxydopamine infusions into the medial forebrain bundle of female Lister Hood rats, resulted in a reduction of usable trials, a near hole bias when animals were tested on the side contralateral to the lesion, as well as increased reaction and movement time latencies. The introduction of an error-correction procedure had no effect on the animals' response bias towards the near contralateral location. Probe trials showed that the bias is most likely the result of responses being misdirected when in a choice situation. The findings further highlight the role of dopamine and an intact striatum to code responses into egocentrically defined space.



## 1. Introduction

Parkinson's disease (PD) is most commonly modelled in experimental animals via infusion of the catecholaminergic neurotoxin 6-hydroxydopamine (6-OHDA) along the nigro-striatal pathway, thereby retrogradely destroying the dopaminergic cells in the ventral mesencephalon, most prominently cells in the substantia nigra (SN) [1–3]. The unilateral lesion is characterised by a profound deficit when animals are tested on the side contralateral to the lesion [1,3–5]. Detailed characterisation of the lateralised deficit by means of operant testing in an operant choice reaction time procedure showed that the deficit is not due to a primary motor or sensory impairment [4,6,7]. Furthermore, when testing was restricted to the side contralateral to the lesion only, via providing lesioned rats a near and a far response location, the lesion caused animals to direct nearly all their responses towards the contralateral near hole, whilst ignoring the contralateral far location. Ipsilateral testing did not reveal the same pattern of deficit [8]. We recently described the phenomenon of unilateral neglect in near complete lesioned animals on a lateralised choice reaction time task that assesses the animals' responses in space on the side ipsilateral and contralateral to the lesion on alternate days [9]. In this study, animals received 6-OHDA lesions to the medial forebrain bundle (MFB) after being trained on this version of the lateralised choice reaction time task. The lesion deficit was stable over

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0166-4328/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.bbr.2012.09.038

more than 50 consecutive days of testing and could only be partly ameliorated by dopamine rich grafts [9]. The lesioned animals maintained a near-hole bias when tested on the side contralateral to the lesion even after the extensive post operative training. The underlying deficit has been proposed to be caused by a distortion in absolute egocentric space representation when tested on the side contralateral to the lesion [8,10,11], i.e. far contralateral responses are misdirected although lesioned rats are capable of detecting and responding to stimuli in the neglected spatial location. Here, we explore the effects of implementing a learning rule that forces animals to respond to the neglected location if they want to obtain a reward. The implementation of this error-correction procedure was thought to be less demanding on working memory and attention mechanisms as animals can perseverate on one hole, until an incorrect response had been made, in which case they could switch the response location in order to obtain a reward.

We have two main hypotheses. Firstly, near complete unilateral lesions via infusion of 6-OHDA into the MFB leads to an impairment in response accuracy in far contralateral space and secondly, the implementation of an error correction procedure does allow for increased accuracy in far contralateral space.

## 2. Materials and methods

All procedures were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act, 1986 and local ethical review at Cardiff University.

### 2.1. Subjects

24 female rats of the Hooded Lister strain (Charles River, UK) were used in the present experiment. All animals weighed 200–225 g on arrival and were housed in groups of 3–4 in standard laboratory cages. The holding room was held at a constant temperature of 21 °C and 50% humidity and a 12:12 h dark–light cycle, with the lights turned on at 7:00 h. Seven days after arrival all rats were placed on a food restriction regime whereby they were presented with weighed amounts of food which was available ad libitum after testing sessions to reduce their body weights to 90% of their free feeding weight. The rats had free access to drinking water during all stages of the experiment.

### 2.2. Procedure

All animals were trained on the lateralised choice reaction time task outlined below (Section 2.5) to asymptotic levels of performance. Rats were then matched into 2 groups based on their overall response accuracy (Control, $n = 10$; Lesion, $n = 14$) before one group received unilateral 6-OHDA lesions to the right medial forebrain bundle. All rats were tested on amphetamine induced rotations 2 and 4 weeks post surgery and at the end of the experiment, before sacrificing for histology. Two days after the last amphetamine session, animals were food restricted again for 7 days before being re-tested on the operant task. After post-lesion data was gathered we conducted a series of probe trials (Section 2.6) before all rats were sacrificed and their brains removed for further histological analysis.

### 2.3. Apparatus

Behavioural testing was conducted in a bank of 12 identical 9 hole operant chambers (Campden Instruments, Loughborough, UK), as described previously [12,13]. In brief, the operant chamber was fitted with a curved array of 9 response holes on one side of the chamber and a magazine on the opposite wall. In the curved array 9 adjacent holes were fitted which could be illuminated and which could record hole entries via breaking of an infrared beam that was vertically monitoring the entrance of the hole. The operant chambers were controlled by the Cambridge Cognition Control BNC (Campden Instruments, Version 1.23) software run on a standard windows desktop personal computer.

### 2.4. Training

7 days after food-restricting the animals to 90% of their free feeding weight (see Section 2.1) animals were gradually trained to perform on the lateral choice reaction time task outlined below (see Section 2.5). All sessions in the operant chambers were conducted over a 30 min session. On the first training day the animals were placed into the box and 20 sucrose reward pellets (45 mg, Sandon Scientific, Hampton, Middlesex, UK) were placed into the magazine. On the second day of training the magazine light was illuminated and a reward pellet was delivered into the magazine. After the rat retrieved the reward, the magazine light was extinguished and the house-light illuminated. After an inter-trial interval (ITI) of 5 s the next pellet was delivered with simultaneous presentation of the magazine light. On the third day of testing a nose-poke into the illuminated centre hole (Hole 5) was rewarded by delivery of a sucrose pellet into the illuminated magazine. After reward collection the magazine light was extinguished and the centre hole light was illuminated again. After 5 days of training all rats were responding highly on this FR1 procedure and were then placed on the choice reaction time task outlined below.

### 2.5. Lateralised choice reaction time task

The lateralised choice reaction time task used has been described in detail elsewhere [8,10]. In brief, animals were trained on different configurations on alternating days, starting always on the side ipsilateral to the lesion. During the respective side of testing, all unused holes were covered by metal well blanks and only three response options were presented: Hole 5 hereafter referred to as centre hole, Hole 4 or 6 (contralateral or ipsilateral), hereafter referred to as near hole, and Hole 3 or 7, hereafter referred to as far hole. At the beginning of each trial the house light was extinguished and the centre light was illuminated. The rat was then required to respond to the centre light by making a sustained nose poke into the hole for a variable delay (50 ms, 100 ms, 150 ms, 200 ms). Upon completion of the delay, a brief stimulus light (300 ms) was randomly presented at either the near or the far response location. When the animal subsequently responded by a nose-poke into the previously illuminated hole it was rewarded by a sucrose pellet into the illuminated magazine. Incorrect responses were "punished" by a time out interval of 5 s during which all lights were extinguished. During initial training all delays were set to 50 ms and the stimulus duration was set to 5000 ms. During subsequent training sessions the delay was gradually increased and the stimulus duration gradually decreased to the standard configuration settings described above.

Importantly, the difference between the present task and previous applications was the implementation of an error-correction procedure. If the animal made an incorrect response the same trial was offered to the animal again after the time out period.

### 2.6. Probe trials

After collecting post lesion data we conducted a series of probe trials to further explore the lesion induced deficit. All probe trials were conducted over 10 days with 5 days testing per side alternating and always starting with the side ipsilateral to the lesion.

#### 2.6.1. Probe 1: Response location shift

The response location was shifted further away from the centre location so that response locations on contralateral days of testing were Far: Hole 2 and Near: Hole 3 and on ipsilateral days of testing Far: Hole 8 and Near: Hole 7.

#### 2.6.2. Probe 2: Stimulus duration

In the second probe trial we kept the configuration of the stimulus-response array as during the probe trial 1 but increased the duration of the stimulus light to 5000 ms.

#### 2.6.3. Probe 3: Simple reaction time near

We tested the animals with only one response option one each side on alternate days. The response locations were Hole 4 on contralateral and Hole 6 on ipsilateral days of testing.

#### 2.6.4. Probe 4: Simple reaction time far

We tested the animals on a simple choice reaction time as described in Probe 3 with the exception that the response locations were furthest away from the centre hole in the stimulus-response array. Therefore, on contralateral days of testing the response location was Hole 1 and on ipsilateral days of testing the response location was Hole 9.

### 2.7. Lesion surgery

Rats of the lesion group received unilateral infusions of the catecholaminergic neurotoxin 6-OHDA (5.41 μg/μl calculated from freebase weight, in 0.2 mg/ml ascorbic acid in 0.9% sterile saline) at a volume of 3 μl into the medial forebrain bundle at stereotaxic coordinates AP: −4.0 mm, ML: −1.3 mm, DV: −7.0 mm, with the incisor bar set at −4.5 (assumed flat head). Infusions were made via a 30-gauge metal cannula which was connected via polyethylene tubing to a 10 μl Hamilton syringe which was driven by a micro drive-pump [14]. The toxin was delivered at a flow rate of 1 μl per minute (=3 min) and 3 min were allowed for the toxin to diffuse in the brain before the needle was carefully retracted. All animals received injections of Metacam® (Meloxicam, Boehringer, Ingelheim, Germany) as analgesic and were placed in heated recovery boxes after surgery.

### 2.8. Rotations

Drug-induced rotations were assessed via i.p. injections of 2.5 mg/kg methamphetamine hydrochloride (Sigma, UK) before animals were placed into rotometer bowls modelled after the design of Ungerstedt [3]. The apparatus recorded net ipsilateral and net contralateral rotations over a period of 90 min distributed into 90

1-min bins. All data were expressed as ipsilateral rotations minus contralateral rotations and averaged over the 90 min session. Therefore a positive rotation score represents rotations towards the side of the lesion.

### 2.9. Histology

Following collection of all behavioural data the rats were deeply anaesthetised with 200 mg/kg sodium pentobarbitone and perfused through the heart with 100 ml phosphate buffered saline (PBS, pH 7.4), followed by 250 ml of 1.5% paraformaldehyde in PBS. Brains were then carefully removed from the skull and placed in the fixative for an additional 24 h after which they were placed in a 25% sucrose solution until sunk.

For visualisation of lesion extent the brains were cut into coronal sections at a thickness of 40 μm on a freezing-sledge microtome into 0.1 M TRIS buffered saline (pH 7.4, TBS). Staining for tyrosin-hydroxylase immunoreactive (TH-ir) cells was done using standard immunohistochemical techniques on a 1 in 6 series of sections:

Coronal sections were first washed in TBS before 5 min quenching endogenous peroxidase enzyme activity in a solution of 3% hydrogen peroxide and 10% methanol in distilled water. After thoroughly washing in TBS (3 × 10 min) they were placed in a blocking solution (3% horse serum in TXTBS) for 3 h after which they were transferred into primary antibody (TH raised in rabbit, polyclonal, Chemicon, 1:1000) overnight. Then sections were washed again in TBS (3 × 10 min) and then incubated for 3 h in biotinylated secondary antibody (anti-rabbit 1:200). After washing in TBS (3 × 10 min) staining was visualised by 2 h incubation in ABC-kit followed by equibrilating the tissue in TNS (2 × 5 min) and evoking the colour reaction by incubation in a 3-3′-diaminobenzadine (DAB, Vector laboratories) solution with 0.3 μl/ml hydrogen peroxide. Stained sections were mounted on gelatine coated slides, airdryed overnight and then coverslipped using a DXP mountant [15].

### 2.10. Statistics

All data were analysed using the GENSTAT v 13.1 software package with Newman-Keuls post hoc testing for multiple comparisons. A significance level of $\alpha = 0.05$ was chosen for all analysis. Operant data was analysed using repeated measures analysis of variance with the factors Group (Control, Lesion), Side (Contralateral, Ipsilateral), Response location (Near, Far), and Week. All data are collated over a 5-day block of testing and response latencies (reaction and movement times) are expressed using geometric means, rather than the arithmetic mean, to reduce the influence of extreme data points.

Main outcome measures are: Trials usable: the number of usable trials where the animal initiated a trial by responding to the illumination of the centre hole by making a nose-poke and sustain that nose-poke for the required duration (delay). Accuracy: the number of correct responses divided by the number of usable trials and expressed as percentage for responses to the near and far response location, respectively. Latencies: reaction (RT) and movement times (MT) for correct and incorrect responses were taken as the latencies from presentation of the lateralised stimulus to withdrawal of the rats' nose from the centre hole (=RT) and from the withdrawal of the nose to executing the response in the response location (=MT). Repeated trials: the number of trials that were repeatedly offered to the animal as a consequence of a previous incorrect response and expressed as percentage of the total number of usable trials. Note that premature withdrawals and omissions do not count towards the number of repeated trials, although the same trial is offered to the rat until a correct response has been made.

## 3. Results

One rat in the lesion group died unrelated to any treatments and therefore the final group sizes were: Control, $n = 10$; Lesion, $n = 13$. No rats were excluded from the lesion group due to lesion size.

### 3.1. Amphetamine induced rotation

There was a significant difference in the average number of ipsilateral rotations between the two groups (Group, $F_{1,21} = 125.29$, $p < 0.001$), with the control group not rotating significantly in any direction ($-0.26 \pm 0.88$ turns/min) and the lesion group exhibiting strong contralateral turning ($12.87 \pm 0.77$ turns/min). The animals' rotational response to methamphetamine increased slightly from $11.8 \pm 0.86$ turns/min to $13.65 \pm 1.03$ turns/min for the lesion group over the three testing sessions, but this marginal increase failed to reach conventional levels of significance (Week × Group, $F_{1,21} = 4.02$, $p = 0.058$). However, whereas controls did not display a rotational bias during any session, lesioned animals rotated highly towards the side of the lesion, therefore indicating a successful lesion.

### 3.2. Immunohistology for tyrosine-hydroxylase

Representative samples of brain sections stained for TH are shown in Fig. 1 for animals of both, the control group (Fig. 1A) and the lesion group (Fig. 1B), respectively. The lesion successfully destroyed the dopaminergic cell bodies in the substantia nigra and largely affected cells located in the VTA as well. Furthermore, this caused a marked depletion in TH-ir staining in the striatum as can be seen in Fig. 1B.

TH-ir cell counts at the level of the medial terminal nucleus of the accessory optical nerve have been used as anatomical borders to separate VTA from SNc cells (Fig. 1C and D). Cell counts in the respective areas ipsilateral and contralateral to the lesion revealed that rats that received lesions displayed a near total (>95%) depletion of TH-ir cells in the ipsilateral SNc (Fig. 1C, Group, $F_{1,21} = 57.44$, $p < 0.001$) and a marked reduction in the number of TH-ir cells in the VTA (60% of contralateral), which was significantly different from control (Fig. 1D, Group, $F_{1,21} = 71.06$, $p < 0.001$).

### 3.3. Operant analysis of behaviour

#### 3.3.1. Trials usable

Before the lesion all animals produced a high number of usable trials (Fig. 2A; mean: $77.2 \pm 1.7$). Rats that received lesions to the MFB produced fewer usable trials than untreated controls in the 5-day block of post lesion testing (Week × Group, $F_{1,21} = 72.12$, $p < 0.001$). All rats produced more usable trials when tested on the contralateral side to the lesion (Side, $F_{1,21} = 86.03$, $p < 0.001$) but this effect was not different between the two experimental groups (Side × Group, $F_{1,21} = 0.09$, $p =$ n.s.).

#### 3.3.2. Accuracy

During baseline performance all animals responded with a high accuracy to the stimulus lights (Fig. 2B and C; Near: 93.35% and Far: 74.13%). The lesion did affect response accuracy differently between the groups, the distance of the response location and the side of testing (Week × Distance × Group, $F_{1,21} = 7.66$, $p < 0.05$). Whereas animals of the control group responded with a similar accuracy to the respective near and far response location during baseline and post lesion, irrespective of the side of testing, animals of the lesion group did show a significant reduction in contralateral far response accuracy (analysis restricted to contralateral: Week × Distance × Group, $F_{1,21} = 61.07$, $p < 0.001$). Whereas on contralateral days of testing response accuracy to the near hole is only reduced to 61.81%, far hole accuracy is reduced to 9.36% correct trials. On ipsilateral days of testing responses to the far hole were less accurate than responses directed towards the near hole (Distance, $F_{1,21} = 26.54$, $p < 0.001$). This difference was not different between the groups (Analysis restricted to ipsilateral; Distance × Group, $F_{1,21} = 3.02$, $p =$ n.s.). Overall ipsilateral accuracy was slightly reduced (−10%) in animals of the lesion group compared to their baseline performance (Week × Group, $F_{1,21} = 11.46$, $p < 0.01$).

#### 3.3.3. Trials repeated

The error correction procedure led to the presentation of the same trial until a correct response had been made. The pattern of the number of trials that had to be repeated on the contralateral side clearly demonstrated the pattern of responding induced by the lesion. Animals of the lesion group had to repeat nearly twice as many trials when the required response had to be made into the far response location, and simultaneous lesioned animals had almost never to repeat a trial that required responding into the near contralateral location (Fig. 2D and E; Weeks × Side × Distance × Group, $F_{1,21} = 4.57$, $p < 0.05$).

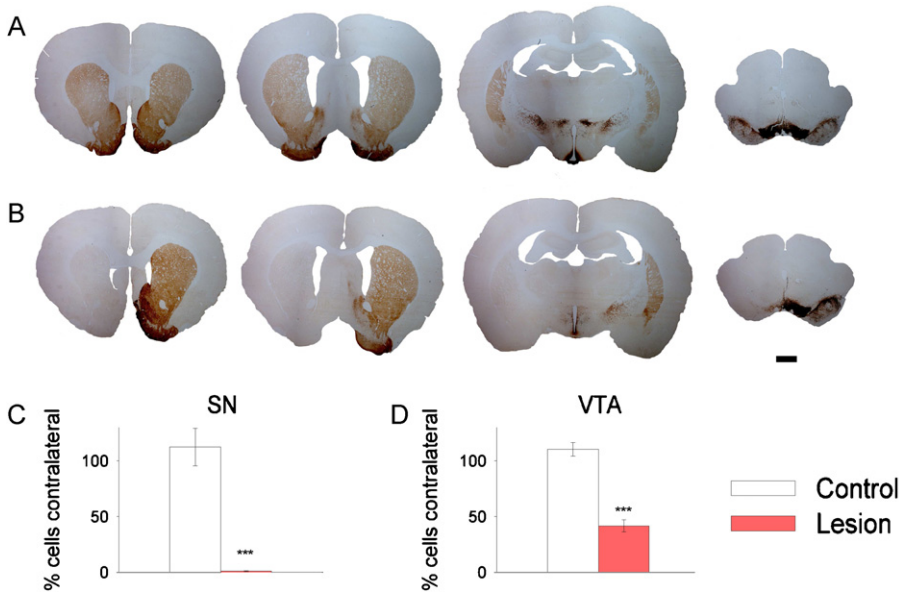


**Fig. 1.** Histological analysis of lesion extent. (A/B) Representative coronal sections stained for TH for both experimental groups. In comparison to animals of the control group (A), rats that received unilateral MFB lesions (B) displayed an absence of TH-ir staining in the contralateral hemisphere throughout the brain. Clearly visible is the TH depletion throughout the striatum as well as the loss of TH-ir cells in the SN. TH-ir cells in the VTA of the lesion group are largely depleted as well. (C/D) TH-ir cell counts at the level of the medial terminal nucleus of the accessory optical nerve expressed as percentage of total cells for the C. SN and D. for the VTA, respectively. Dotted line represents no bias. Asterisks denote significant different from control at $p < 0.001$ and Scale bar represents 1000 μm.

*3.3.4. Reaction time*

The time to react to the occurrence of the lateralised stimulus light was increased in lesioned animals after the surgery (Fig. 2F and G; Week × Group, $F_{1,21} = 26.18$, $p < 0.001$). Reaction time was only increased when testing was conducted on the side contralateral to the lesion (Side × Group, $F_{1,21} = 30.41$, $p < 0.001$). There was no difference in time to react to stimuli in either the near or the far response location (Distance, $F_{1,21} = 1.06$, $p =$ n.s.).

*3.3.5. Movement time*

The time to respond to the lateralised stimulus was longer for far hole responses than for near hole responses (Fig. 2H and I; Distance, $F_{1,21} = 17.34$, $p < 0.001$). This effect was not different between the groups (Distance × Group, $F_{1,21} = 1.31$, $p =$ n.s.) and not between the weeks of testing (Week × Distance, $F_{1,21} = 1.34$, $p =$ n.s.). Instead, similar to reaction time responding, the time to execute the response was increased on contralateral days of testing in animals that received lesion surgery (Week × Side × Group, $F_{1,21} = 24.11$, $p < 0.001$). Although movement times were increased in the lesion group on ipsilateral days of testing (Ipsilateral: Week × Group, $F_{1,21} = 15.95$, $p < 0.001$), the increase in movement time was much more pronounced on contralateral days of testing (Contralateral: Week × Group, $F_{1,21} = 43.94$, $p < 0.001$; Overall: Side × Group, $F_{1,21} = 25.36$, $p < 0.001$).

*3.4. Probe trials*

All probe trials were conducted over 10 consecutive days of testing. During probe trials 1 and 2 testing was conducted on alternate days whereas during probe 3 and 4 testing was conducted over 5 consecutive days/side, always starting ipsilateral.

*3.4.1. Shift of response location*

During the first probe trial we shifted the response options for one location further away from the centre hole, thereby making the former far hole the near hole. The animals' performance on this configuration was similar to testing under the standard configuration used during baseline and post lesion testing (Fig. 3A). Although near hole accuracy was reduced for the lesion group (48.54% during Probe compared to 61.81% during Standard, post lesion configuration), lesioned rats displayed a similar accuracy pattern with higher response accuracy towards the near response location with a response accuracy score of 2.28% towards the far contralateral location (Distance × Side × Group, $F_{1,21} = 8.83$, $p < 0.01$). Important to note is that lesioned rats now respond to exact same spatial location that was neglected previously.

*3.4.2. Shift of response location and increase in stimulus duration*

The configuration of the response holes was as described above (Section 3.4.1) but in an attempt to facilitate responding the stimulus duration was set to 5000 ms. As can be seen in Fig. 3B lesioned rats did increase response accuracy to the contralateral near hole when compared to the configuration used in Probe 1. This increase in accuracy only affected the near contralateral response location; when responses had to be directed towards the contralateral far hole, animals still chose to ignore this spatial location (Distance × Side × Group, $F_{1,21} = 174.62$, $p < 0.001$).

*3.4.3. Simple reaction time – near configuration*

After assessing the effects of the lesion on the 2-choice version we tested all animals on a simple reaction time task with only one response location. During this assessment only the centre hole and the hole immediately adjacent was available to the rat (i.e. Hole 4 and Hole 6, respectively).

Here, the difference in contralateral accuracy was too small to cause a significant difference between the two experimental groups (Fig. 3C; Sides × Groups, $F_{1,21} = 4.05$, $p = 0.057$), whilst, overall, the lesion group did perform slightly under the control group (Group, $F_{1,21} = 6.81$, $p < 0.05$).

*3.4.4. Simple reaction time – far configuration*

After assessing the effects of removing one response option on the simple near configuration we assessed the same effect when the single response option was located furthest away from the centre hole.

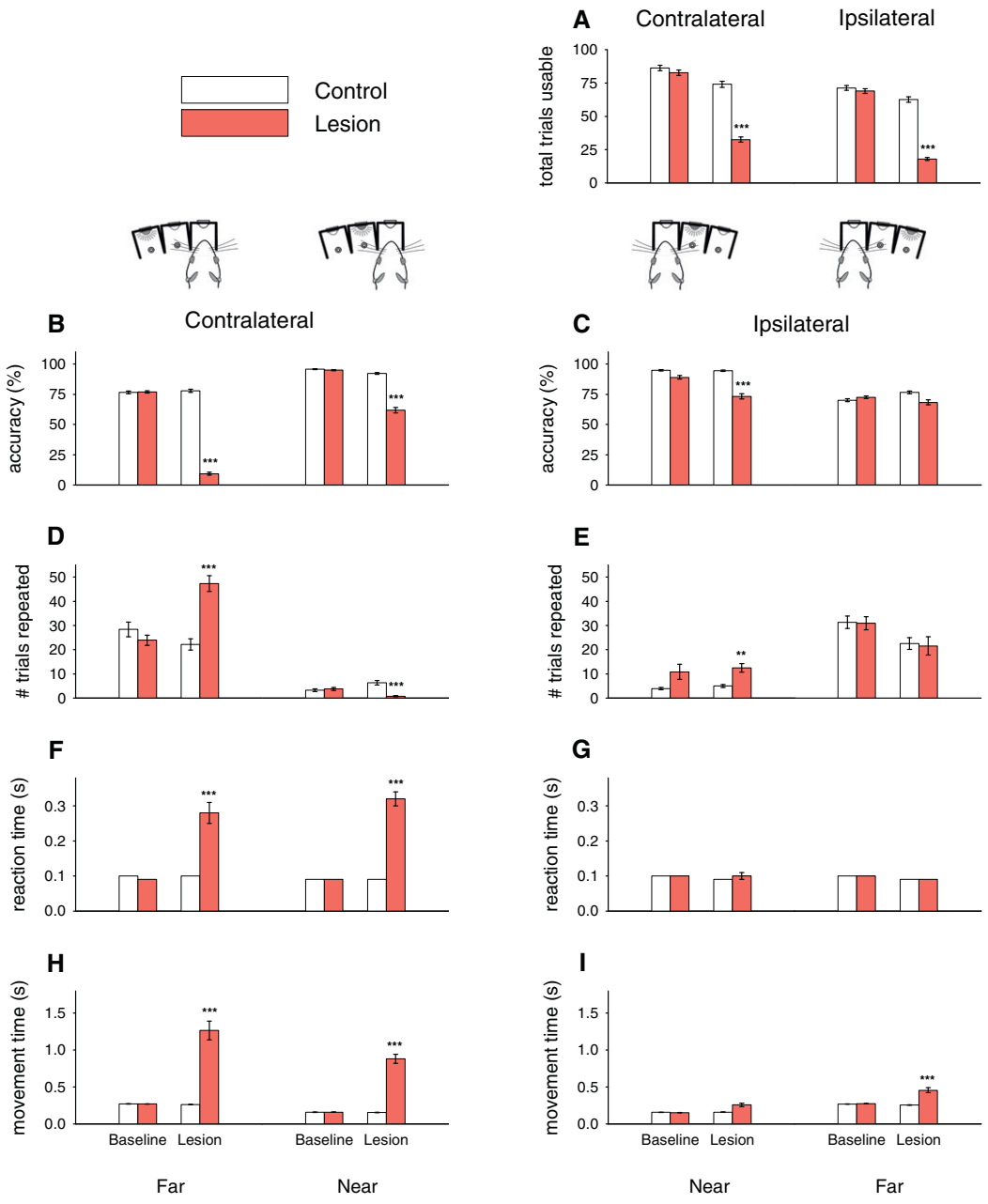


**Fig. 2.** Main effects of the choice reaction time task. Data are presented for the number of usable trials per side of testing (A) and split for responses ipsilateral (B, D, F, H) and contralateral (C, E, G, I) to the lesion for lateralised parameters and for each side responses are shown for the near and far response location, respectively. The illuminated hole depicted in the schematic above the graphs for contralateral and ipsilateral responses (B-I) indicate the correct response required. Asterisks denote significant different from control at the respective timepoint/response location at $p < 0.01$ or $p < 0.001$ for two or three asterisks, respectively.

All lesioned animals displayed a contralateral deficit in response accuracy with performance similar to control when tested on the ipsilateral side (Fig. 3D; Side × Group, $F_{1,21} = 19.64$, $p < 0.001$). Although the animals had to respond to the furthest possible location in our stimulus array, contralateral accuracy was still 42.38%.

## 4. Discussion

The version of the choice reaction time task used in the present experiment revealed differences in response patterns between lesioned and untreated control animals and further elucidates the role of dopamine depletion in the striatum. Rats that received unilateral lesions to the MFB displayed reduced responding to the contralateral far hole whilst leaving ipsilateral responding largely intact. Probe trials showed that the lesioned animals did not have an allocentric response deficit as (i) lesioned rats did respond with high accuracy towards ipsilateral near and far stimuli and contralateral responding was largely biased towards the nearer of two response locations; (ii) lesioned rats were able to respond to the previously neglected spatial location when the response options were shifted, i.e. the default response profile was to respond to the most proximal location in contralateral space, irrespective of the absolute spatial position, when faced with a response choice. Reducing task demands increased overall accuracy but did not alleviate the response bias in lesioned rats. Interestingly, the described deficit was only apparent when the rats were faced with a choice discrimination. When we conducted probe trials where rats had to respond to only one stimulus on one side of the rats' head (simple reaction time task), there was no difference between lesioned rats and those of the control group. Furthermore, even when tested in

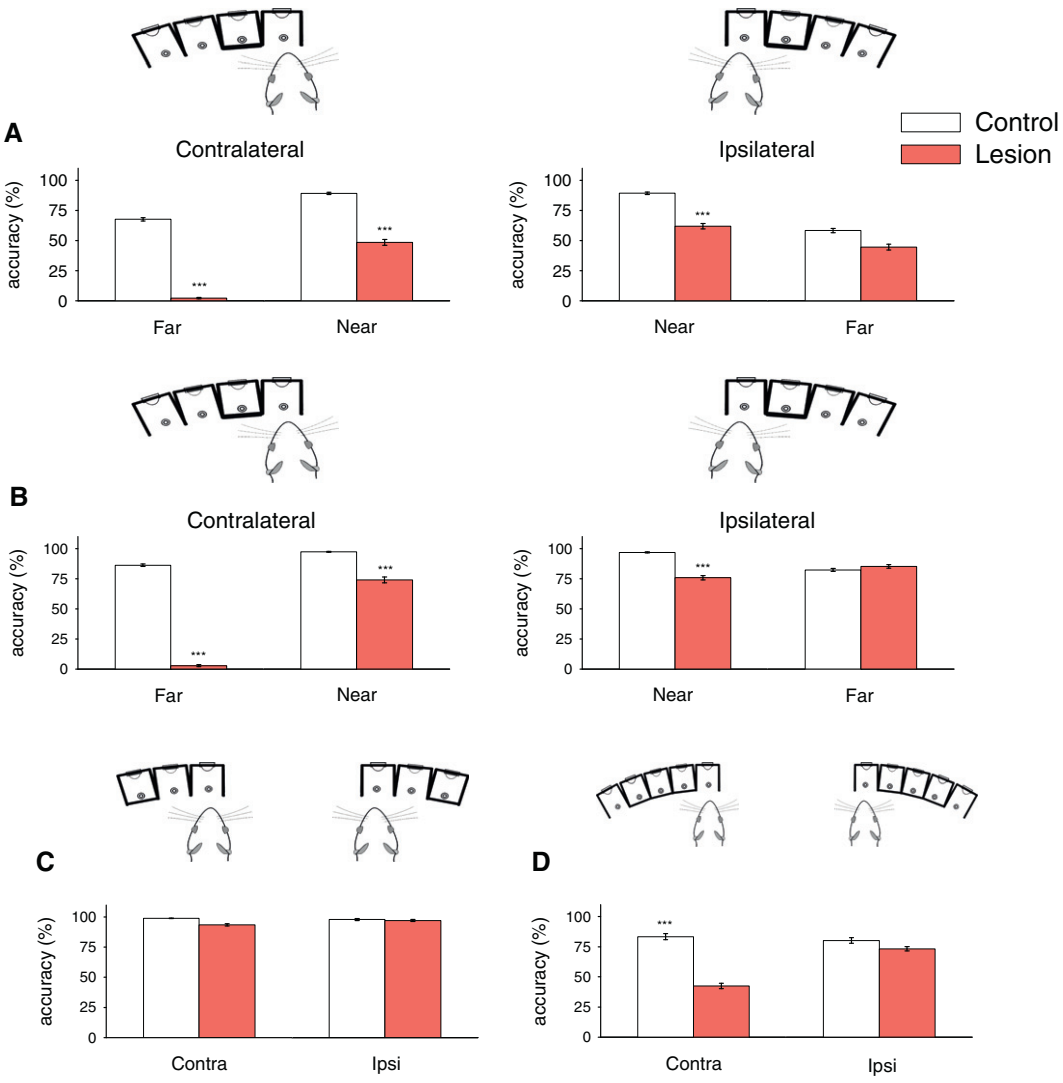


**Fig. 3.** Percentage of correct responses when rats were tested on the different probe trials presented per side of testing. (A) Probe 1: the location of the response hole was shifted further away form the centre hole. (B) Probe 2: the configuration of the response location was kept as under Probe 1 and the presentation of the stimulus light was increased from 300 ms to 5 s. (C) Probe 3: the configuration was changed to a simple reaction time task with the only lateralised response option being the hole immediately adjacent to the centre hole. The data shows the percentage of correct responses when rats were tested Probe 2. Data are presented for ipsilateral and contralateral responses to the near and far response location, respectively. (C) The data shows the percentage of correct responses when rats were tested on Probe 3. Data are presented for ipsilateral and contralateral responses respectively. (D) The data shows the percentage of correct responses when rats were tested on Probe 4. Data are presented for ipsilateral and contralateral responses respectively. Asterisks denote significant different from control at the respective time point/response location at $p < 0.01$ or $p < 0.001$ for two or three asterisks, respectively.

the most extreme/distal configuration (Probe 4), lesioned rats displayed lower response accuracy than control group animals when tested on the contralateral side, but importantly, they responded much higher than during any of the far holes in all other tests, which were spatially closer to the rats starting position. Based on this, and findings from previous studies utilising partial lesion models, we can conclude that the contralateral neglect that is caused by striatal dopamine depletion is not due to a primary impairment in sensory or motor processing [4,6–8,16,17].

The deficit seen in rats on this version of the choice reaction time task introduced by Brown and Robbins (1989) confirmed the deficit of unilateral egocentric based neglect in contralateral space that has been reported after partial dopaminergic depletion [8] as well as after excitotoxic lesions of the striatum [10,18]. Here we report for the first time that near complete lesions produce similar deficits. The near complete lesion model via unilateral 6-OHDA infusions to the MFB is the most commonly used model to study the effects of cell replacement therapies. Whereas partial lesions, via infusions of the neurotoxin to the terminals in the striatum, are more variable and prone to spontaneous recovery, the current model produced stable behavioural deficits [1,6,7,13,19]. The assessment of behaviour by means of operant testing has been shown to be more sensitive to the effects of therapies than tests of simple motor asymmetry [13]. The novel description of the version of the choice reaction time task that is employed in the present study allows for a more in-depth analysis of the effects of cell replacement therapies in the future.

Indeed because of previous findings that performance is only impaired in a choice discrimination setting, the introduction of an error correction procedure was implemented to encourage animals to respond into far contralateral space in order to obtain a reward. We speculated that the development of a near hole bias could be due to a reduction in motivational drive and/or the development of a alternate response strategy induced by the lesion. In a Skinner box version of the choice reaction time task it became clear that lesioned animals would respond to all stimuli by turning in the same direction after the hold period had elapsed. They would respond directly by turning towards the ipsilateral side to make the

response or conduct a full body turn to execute the response to the lever located contralateral to the lesion. Therefore, we speculated if the contralateral near hole bias was a result of a changed response strategy. An alternative explanation would be that lesioned animals display a lowered motivational state [20] as we and others have shown that the dopamine depletion causes a marked reduction in the number of trials attempted [7,13,21]. Indeed, if animals would only respond towards the near hole, the task demand on working memory and attention would be lower than when making choices. Although they would not get rewarded when the required response had to be made towards the far contralateral response location, they still received a reward on 50% of all trials as the chance of a correct near hole response was randomly determined by the computer. The implementation of an error correction procedure was thought to prevent this strategy as perseveration towards the near hole would not lead to any rewards.

In contrast to our initial hypothesis, the error correction procedure was not effective in forcing the animals to respond to the far response location on contralateral days of testing. As shown by the number of trials repeated, lesioned rats persevered responding to the contralateral near hole. Shifting the response location on this version of the task revealed, as previously [8,9], that rats are capable of responding to this specific spatial location and that the deficit shifts to the new "far" hole, whilst responding with high accuracy to the, now "near", response location that formerly had been neglected. Increasing the stimulus length to 5000 ms, longer than the time needed by the animals to execute the lateralised response, had only an effect on contralateral near hole accuracy. Although rats could have used the stimulus light as a beacon to guide their responses they continued to direct almost all response towards the near contralateral location.

Of interest are the results of the simple reaction time trials which show that, although accuracy was lower on contralateral trials in the far condition, lesioned animals still responded with high response accuracy. Furthermore, a contralateral accuracy deficit was not found when the response locations were most proximal to the animals' head in a simple reaction time setting (Probe 3), whereas a more lateralised deficit was found when the response location was set most distal (Probe 4).

The contralateral far hole neglect was only present when animals were presented with a response choice, but not when responding was limited to one location only in near space. Previous work has shown that accuracy on a simple reaction time was not differently affected by striatal lesions than accuracy on a choice reaction time task [17]. This is in contrast to the findings presented above. The main difference between the two studies is that whereas we present the animals with a true simple reaction time task, with only one response option within session, the latter study used pre-cueing as a means to provide all information necessary to the animal [17]. It has been argued that fully cued tasks are different from simple reaction time tasks [22], and only when enough time is given pre-execution of the response similarities can be considered [22]. Still, demands on working memory would be higher to remember the cue compared to a situation when only one response is possible.

Our results support previous findings of striatal dopamine depletion and cell body lesions of the striatum on this type of choice reaction time task. The accumulating data show, that after a lesion, responding in far contralateral space is impaired and that this impairment is due to a failure in directing responses towards the correct location [8,10]. Previous research has shown that when the two striata are put in competition, by means of presenting both or neither stimulus simultaneously, lesioned animals display a near hole bias when tested on the contralateral side of the lesion, whereas no bias was recorded when no stimulus lights were presented [8]. Striatal cell body lesions caused the same egocentric contralateral neglect as discussed above [10] but the rats' behaviour on a series of probe trials revealed interesting functions of the striatum. By means of shifting the response locations around the centre hole whilst leaving the absolute distance between both locations constant they showed that the deficit is greater when contralateral location is furthest away when compared to the mirrored configuration (Near contra vs. far ipsi) [10].

In conclusion, we have shown that the response bias induced by unilateral near-complete dopamine depletion in rats does not recover by implementation of an error correction procedure that "encourages" the animals to respond towards the far stimulus configuration in a choice reaction time setting. These findings further highlight the role of dopamine in the facilitation of directing responses into egocentrically coded response space.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

This study was supported by the European Community's Seventh Framework Programme NeuroStemCell, under grant agreement nr. 222043, and the Medical Research Council.

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# Chapter 3.4

## Experiment 4: Unilateral nigro-striatal 6-hydroxydopamine lesions in mice I: Motor impairments identify extent of dopamine depletion at three different lesion sites

The majority of studies applying the 6-OHDA lesion to model PD have been conducted in rats. Although initial studies utilised mice, only recently the mouse model has become more popular. Major challenges in injecting mice with the DA depleting toxin have been the high mortality rate in complete lesioned animals and the high variability in lesion success.

Recent work from Sweden in Angela Cenci-Nilsson's group and others have shown that by implementing an extensive caring protocol, mortality rates can be reduced to acceptable levels. Furthermore with the advent of mouse-derived stem cells and genetic manipulations the lesion mouse model is of interest for many neuroscientists. Although some of the lesion models have been investigated individually, i.e. MFB, or SN lesions, no study, to our knowledge, has attempted a direct comparison of lesion procedures on pathological changes in the brain or behavioural measures. Moreover, to date the behaviour conducted in mice is limited and mainly focuses on translations from the rat. Only the recent study by Grealish et al. (2011) – which was published after the present study had been completed - has attempted to characterise the behavioural sequalae of unilateral SN lesions, where the behavioural deficit was correlated with the extent of the lesion and provided first guidelines for a distinction between well lesioned from partially lesioned animals. The aim of the present experiment was to conduct a direct comparison of all three unilateral lesion models via infusion of 6-OHDA along either, the SN, the MFB, or the striatum and assess the lesion induced behavioural changes on a wide panel of behavioural tests.

The experiment conducted in the present paper as well as analysis of the data, histology and preparation of the manuscript was undertaken by myself in close collaboration with a fellow PhD student, Gaynor Ann Smith, who is joint first author of the resulting publication presented below and the complementary paper (Smith et al. 2012). Professor S.B. Dunnett, Dr. Lane and Dr. Lelos were involved in planning of the experiment and gave help and advice throughout as well as in the writing of the manuscript.

Behavioural Brain Research 228 (2012) 30–43

Contents lists available at SciVerse ScienceDirect

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Research report

# Unilateral nigrostriatal 6-hydroxydopamine lesions in mice I: Motor impairments identify extent of dopamine depletion at three different lesion sites

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ARTICLE INFO

*Article history:*
Received 30 July 2011
Received in revised form
17 November 2011
Accepted 21 November 2011
Available online 28 November 2011

*Keywords:*
Parkinson's disease
6-Hydroxydopamine
Mouse
Motor behaviour
Dopamine
Striatum

ABSTRACT

The unilateral 6-hydroxydopamine mouse lesion models of Parkinson's disease have received increasing attention in recent years, but comparison of the different lesion models was largely focused at a histological level. An extensive behavioural comparison between different mouse models on tests of motor function has yet to be carried out, to pin point tests that accurately discriminate between different extents of dopaminergic depletion. In the present study we examine the consequences of injection of the toxin at three sites along the nigrostriatal tract (substantia nigra, medial forebrain bundle, and striatum) on a broad range of simple motor tasks, and on the dopaminergic pathology. All lesion groups demonstrated marked behavioural deficits and displayed distinct profiles of degeneration along the nigrostriatal dopamine pathway. Tests that correlated closely with the level of substantia nigra cell loss included the corridor, cylinder and balance beam tests, the rotarod, inverted cage lid and three types of rotational assessment (spontaneous, amphetamine-induced and apomorphine-induced). Specific tasks are identified which are capable of distinguishing a near-complete lesion, with amphetamine rotation, corridor and cylinder tests showing the highest correlations with levels of nigral cell loss. Performance in the different behavioural tests was associated with distinct profiles of cell loss in the SN and VTA. We provide a comprehensive behavioural assessment of lesion-induced deficits in mouse models of PD, which should facilitate selection of the most appropriate lesion model and most sensitive behavioural tests for use in future studies investigating therapeutic interventions.



## 1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterised by resting tremor, bradykinesia and postural instability [1]. Post mortem analyses of Parkinsonian brains have revealed that substantial degeneration of the pigmented dopaminergic cells in the ventral midbrain is the likely cause of many of the motor features [2,3] and extensive degeneration of nigral dopaminergic neurons is needed before the first overt motor symptoms occur [4].

Since the first description of the 6-hydroxydopamine (6-OHDA) lesion rat in 1968, injection of this catecholaminergic neurotoxin has been widely used to model the nigrostriatal dopamine depletion in PD [5,6]. Intraventricular injection of 6-OHDA in the rat produces a bilateral catecholaminergic depletion with consequential motor impairments and profound impairments in feeding, drinking and ability of the animal to maintain full health [7]. Unilateral administration avoids these health problems and creates a unilateral motor impairment, the extent of which can be determined using a variety of behavioural tests. In the rat, the most complete unilateral lesions are achieved via injection of the toxin into the medial forebrain bundle (MFB) where the ascending nigrostriatal fibre pathway is at its most compact. Alternative sites can be used along the nigrostriatal trajectory, directing the toxin to either the cell bodies in the substantia nigra (SNc), or the terminal region of the fibres in the striatum, where the dopaminergic terminals of the nigrostriatal pathway undergo massive ramification and make synaptic contact with their primary targets, the medium spiny neurons. These approaches have the advantage of permitting more selective nigrostriatal lesions, avoiding the ventral tegmental area projections involved in the limbic circuitry. From studies in the rat we know that these different lesion profiles can produce distinct behavioural deficits, reviewed in Refs. [8,9]. Now, with the increasing use of transgenic mice in experimental therapeutics, a comparable understanding of the deficits and their dissociation after different lesions in mice is needed to enable selection of effective lesions, assessment of pharmacotherapeutics and evaluation of non-drug mediated treatment strategies such as cell replacement.

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doi:10.1016/j.bbr.2011.11.027

Given that all the original lesion work and characterisation of motor tests was carried out in the rat, developing and optimising a similar lesion model and translating the now well-established behavioural tests into mice has faced significant challenges [10–14]. A comparison of the available literature shows variable results across studies, particularly in the use of drug-induced rotation (e.g., following peripheral injections of apomorphine or amphetamine) as a screening procedure for the extent of dopamine depletion [13,15,16]. Many of the standard tests, which are reliably used in the rat appear to be less discriminative in the mouse. Practical problems in lesioning mice include very high mortality rates when the toxin is aimed at the MFB, reaching >80% in some studies [10,13], which has led some groups to prefer nigral or striatal lesions. A direct comparison between the three different lesion models has been described recently with regards to lesion effects on histopathology and in vivo assessment of locomotor activity, rotational response and paw preference [17], but there has yet to be a comparison between the three lesion models on a wider array of behavioural tests and an explicit attempt to identify behavioural changes indicative of the extent of the lesion. The aim of the present study was to directly compare the relationship between the extent or pattern of unilateral dopamine depletion and behavioural impairment, to identify robust behavioural assays for selecting mice with the most complete lesion. Criteria for behavioural tests should be that they allow for high throughput screening, simple test administration and lesion-induced impairments that correlate with nigral cell loss. We therefore employed a comprehensive range of the most popular motor tasks after each lesion type, in order to determine the most reliable tests for identifying distinct lesion profiles. After analysis of the behavioural deficits induced by each lesion on simple motor tests, the relationship between the magnitude and pattern of dopamine depletion and behavioural performance was evaluated allowing identification of the tests that are most sensitive to the presence and extent of dopamine depletion.

## 2. Materials and Methods

### 2.1. Subjects

A cohort of 105 male C57/Bl6 mice (Charles River, UK) aged 10–12 weeks on arrival were housed in groups of 5–6 in standard laboratory cages with food and water ad libitum, at 21 ± 1 °C room temperature, 60 ± 1% relative humidity, and a 12:12 h dark–light cycle with lights on at 06:30 h. All testing was conducted between 08:00 h and 17:00 h. For the tests that utilised sucrose reward pellets, mice were food restricted for 7 days prior to testing, maintaining them at 90% of their free feeding weight, and with free access to drinking water at all times. All mice received weighed quantities of food, which was then placed into the home cages for consumption. All experiments were conducted in compliance with the UK Animals (Scientific Procedures) Act 1986 and approved by local ethical review.

### 2.2. Experimental design

After pre-lesion training on the staircase test (see Section 2.4.1), mice were allocated into four matched groups based on the number of pellets eaten and the presence of a side bias. One group ($n = 15$) served as untreated experimental controls that did not undergo any surgical procedure, whilst the other three groups ($n = 30$ in each) underwent surgery. Unilateral lesions to the dopaminergic system were aimed at the SNc, MFB, or striatum. Six weeks post-lesion, mice were food-restricted and tested on the staircase test and corridor test. Mice were allowed ad libitum access to food for the remainder of the experiment. The following behavioural tests were then conducted consecutively: balance beam, spontaneous rotation, cylinder, rotarod, stepping, gait, inverted cage lid, locomotor activity and separate amphetamine- and apomorphine-induced rotation sessions (Fig. 1).

### 2.3. Lesion surgery

Mice were anaesthetised in an induction chamber with 1.5–2.0% isoflurane using oxygen as the carrier gas. The top of the head was shaved and the mouse was placed into a Kopf stereotaxic apparatus providing 1.5–2.0% isoflurane anaesthetic in 2:1 oxygen/nitrous oxide carrier gas. For all three lesion types the incisor bar was set at the level of the interaural line. Unilateral lesions were achieved by injection of 6-OHDA (Sigma, Poole, UK) via a 30-gauge stainless steel cannula connected to a 10 μl Hamilton syringe mounted on a microdrive pump via polyethylene tubing. The neurotoxin was used at a concentration of 6 μg/μl (hydrobromide salt, calculated from free base weight) dissolved in a solution of 0.9% sterile saline in 0.2 mg/ml ascorbic acid. The cannula was targeted at the right SNc (1.5 μl), MFB (1 μl), or striatum (2 × 1.5 μl), at the following stereotaxic coordinates (relative to bregma, in mm): SNc: AP = −3.0, ML = −1.2, DV = −4.5; MFB: AP = −1.2, $L$ = −1.2, DV = −4.75; and striatum: (i) AP = +1.0, $L$ = −2.1, DV = −2.9; (ii) AP = +0.3, $L$ = −2.3, DV = −2.9. The required volume was injected at a rate of 1 μl/min into each target, following which, the cannula was left in place for 3 min to allow for diffusion before retraction of the cannula, cleaning and suturing the wound. At the end of each surgical session mice were given a subcutaneous injection of 0.5 ml of 0.9% sterile saline containing 4% glucose daily (14 days), along with soluble paracetamol analgesia in the home cage water bottle (1 g/l for 3 days) and provision of wet granulated food. Body weights were monitored for 14 days post lesion. If the weight dropped below 85%, the health of the animal was evaluated and the mouse was sacrificed if considered appropriate. All mice were given access to free food and time for recovery for a total of 5 weeks post lesion before food was withdrawn 7 days prior to the commencement of post-lesion testing (Fig. 1).

### 2.4. Behavioural tests

Prior to each behavioural test, mice were habituated to the testing room for at least 20 min. For the staircase test and the corridor test mice were food restricted for 1 week prior to testing. Assessments on all other behavioural tests were conducted after mice had been allowed ad libitum access to food for at least 7 days. Behavioural tests were conducted in the order outlined in Fig. 1 and as described below.

#### 2.4.1. Staircase

The staircase test for mice has been adapted from the rat procedure as an objective test of skilled forelimb use [14,18]. A detailed description of the mouse staircase apparatus (Campden Instruments, Loughborough, UK) can be found in Ref. [14] and illustrated in Ref. [19]. In brief, a short corridor off the start box is fitted with a central plinth onto which the mouse can climb and a narrow gap is present on either side. On either side of the plinth there is a staircase with eight equal steps. Each step is baited with 2 precision sugar pellets (20 mg, Sandown Scientific). The apparatus is configured such that the mouse can only reach and retrieve pellets from each staircase with the corresponding forepaw on that side, so that the total number of pellets removed provides a measure of reaching success independently on each side of the body.

On the first day of training, both staircases were baited with excess pellets and additional pellets were scattered on the plinth of the platform, and food-restricted mice (see Section 2.1) were placed in the staircase apparatus for 20 min to explore. After this initial day of habituation, all mice were tested for 30 min over seven consecutive days during which mice' performance reached asymptote. The last 3 days of asymptotic performance were then collated as baseline (pre-lesion) performance. Post-lesion data is similarly collated over a 3-day block. For statistical analysis, reaching deficits are expressed in terms of the number of pellets retrieved on the left (impaired, contralateral to lesion) side as a percentage of the number retrieved on the right (control, ipsilateral to lesion) side.

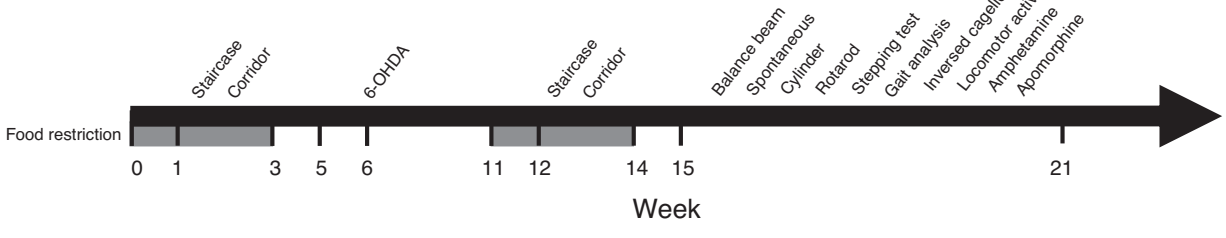


**Fig. 1.** Experimental plan by week of testing. Areas of grey indicate periods of food restriction.

*2.4.2. Corridor*

The corridor task assesses lateralised sensorimotor proprioception and neglect, and was adapted for use in the mouse based on previous studies in the rat [13,20]. The apparatus was custom built to have two adjacent corridors 60 cm long, 4 cm wide and 15 cm high. Ten pairs of adjacent pots, each with a diameter of 1 cm and containing six 20 mg sugar pellets (Sandown, USA), were placed in one corridor, equally spaced at 5 cm intervals. The adjacent corridor was used to acclimatise mice to the equipment before the testing session. Food restricted mice (see Section 2.1) were tested on the corridor apparatus for three consecutive days, pre- and post-lesion. Mice were habituated to the apparatus for 20 min on two consecutive days prior to testing. On the third day (test day) the number of right and left successful retrievals made by each mouse was counted until a total of 20 retrievals were recorded, or until 5 min elapsed. A % side bias score was calculated (100 × (left retrievals)/(total retrievals)) and used for the statistical analysis.

*2.4.3. Balance beam*

The beam test analyses the motor coordination of hindlimbs and forelimbs during precise locomotion and balance, and has been shown to be sensitive to unilateral 6-OHDA lesions in the rat [21]. Here, the beam has been adapted such that the height, 17° incline and converging width of the beam encourage the mouse to ascend from the start point to an enclosed box located at the uppermost and narrowest part of the beam (illustrated in Smith and Heuer [19]). Beam dimensions are: *L*80 cm, *W*0.5–1.5 cm, *H*34–54 cm. House dimensions are: *L*11 cm, *W*11 cm, *H*10 cm. The training protocol for the balance beam has recently been described in Ref. [22]. Briefly, all mice were tested on the balance beam apparatus for three consecutive days. During the first 2 days the mice were trained to traverse the beam by firstly habituating them to the goal box and secondly, moving them to the starting point on successive trials further away from the entrance of the goal box until the mouse was able to traverse the entire distance of the apparatus. On the third (test) day, each mouse was video-taped on three consecutive trials and the time taken to ascend the beam and the total number of hindlimb and forelimb foot slips were counted on each trial. The two quickest times and foot slip counts were averaged. Contralateral foot slips were scored during the trials and ipsilateral foot slips were scored post hoc from video footage. A foot-slip side bias score was calculated (as above, Section 2.4.2) for each mouse.

*2.4.4. Cylinder*

The cylinder test assesses the laterality of forelimb use in a novel environment [23,24]. Mice were tested for paw preference in a spherical glass beaker with a diameter of 11.5 cm, illustrated in Ref. [19]. Two mirrors were positioned at 60° and placed directly behind the cylinder, which ensured a 360° view was available to the observer. Each mouse was allowed to make 20 rears with weight-bearing contact against the side of the cylinder and the left/right forelimb bias was recorded. The session was videotaped and scored by detailed freeze frame analysis. Simultaneous ipsilateral and contralateral forelimb touches were excluded and mice that failed to reach 20 touches were removed from the cylinder after 10 min. Data are expressed as side bias of paw preference (calculated as above, Section 2.4.2). All mice were tested only once on the cylinder test to prevent habituation to the apparatus.

*2.4.5. Rotarod*

The rotarod apparatus (Ugo Basile, Varese, Italy) was used to provide an overall assessment of balance, motor coordination and strength [23–26]. Mice were tested over three consecutive days with two training days of 3 trials at 12 rpm and 22 rpm, respectively. Trials lasted 300 s and were separated by at least 1 h to prevent fatigue. During training, mice that fell off the beam were placed back on until the 300 s had elapsed. On the test day mice were tested three times on an accelerating protocol in which the rotation speed was increased from 4 to 44 rpm over 300 s. The time to fall off the beam was recorded as the outcome measure and an average of the two best trials was used for statistical analysis.

*2.4.6. Stepping test*

The stepping test [27] was conducted on one testing day using a modified protocol for mice [28]. In short, mice were placed on a flat table and left there to settle for 3 s. Subsequently the hind legs were elevated from the surface by lifting the mouse by the base of its tail and gently pulling it backwards over a distance of 50 cm over 5 s. Video recordings and frame-by-frame playback were used to count the number of adjusting steps the mouse made. All mice were tested three times and the average number of adjusting steps for each side was expressed as bias score (Section 2.4.2).

*2.4.7. Gait analysis*

The animals' gait was analysed using the footprint method by which the fore- and hind-paws were differently coloured using non-toxic water-based paint [29,30]. Testing was conducted on one day in the corridor apparatus, which encouraged the mice to run in a straight line (see above for dimensions) and white absorbent paper was laid down to record the footprints. The distance covered by three consecutive strides was taken as the outcome measure.

*2.4.8. Inversed grid test*

Mice were placed onto the centre of a metal home cage lid that was taped off around the edges, allowing access to a rectangle of approximately 20 cm × 21 cm [31–33]. The cage lid was then carefully inverted and secured in a stable position at a height of 30 cm. To dampen any falls, the area under the cage lid was covered in towels. Testing of all mice was conducted on one day across three sessions, which were separated by at least 1 h to prevent fatigue. The duration of time the mouse spent grasping the grid without falling off was recorded, with a maximum of 300 s. The average of the two best times was used for the statistical analysis.

*2.4.9. Activity cages*

Locomotor activity was monitored over a 2 h period during the light phase using an automated system (Med Associates, St Albans VT, USA, with MED-PC IV software). Mice were placed in individual Perspex cages with three infrared beams crossing the base of each box (dimensions: *L*42 cm, *W*26 cm, *D*19 cm). The total number of non-perseverative beam-breaks was automatically recorded.

*2.4.10. Rotation*

Spontaneous rotation in a novel spherical environment was assessed using video footage from the cylinder test. Net rotation during the first 5 min habituation phase was scored. This test was based on open field observations showing that mice turn towards the side of the lesion when placed into a novel environment [10,12,34]. Amphetamine- and apomorphine-induced rotations were tested on two separate occasions with the apomorphine test being conducted 1 week after the amphetamine-induced rotation. During testing, mice were video-recorded from above and rotational scores were scored post hoc [15,35,36]. Drug-induced rotation has previously been validated in the lesion mouse model [15]. Each mouse was placed in a glass cylinder and was videotaped using an overhead camera. Full turns were counted in the ipsilateral and contralateral directions during a 20 min window of peak rotational response and data are expressed as net rotations (20–40 min after injection of D-amphetamine sulphate (2.5 mg/kg in 0.9% saline i.p.); 5–25 min after injection of apomorphine hydrochloride (0.05 mg/kg in 0.9% s.c.).

*2.5. Immunohistochemistry*

After completion of behavioural testing the mice were terminally anaesthetised with sodium pentobarbital and transcardially perfused with approx. 25 ml phosphate-buffered saline (PBS), followed by approx 100 ml 1.5% paraformaldehyde (PFA, pH 7.4 in PBS). Brains were dissected from the skull and post-fixed in 1.5% PFA for 24 h before being cryoprotected in a 25% sucrose solution. Coronal sections were then cut at 40 μm thickness using a freezing microtome and stored at 4 °C in PBS with 0.01% azide. Standard immunohistochemical techniques were used to visualise dopaminergic innervation of the striatum as well as cell bodies within the substantial nigra. In brief, sections were washed thoroughly in PBS before quenching of endogenous peroxidases using 10% methanol and 3% hydrogen peroxide for 10 min. After washing with Tris-buffered saline (TBS), the sections were incubated in 5% normal horse serum (NHS) with 0.25% Triton X-100 dissolved in TBS (Tx-TBS). Tissue sections were then incubated in primary antibody for 16 h at room temperature at concentrations of 1:1000 tyrosine hydroxylase (TH, polyclonal raised in rabbit, Chemicon International, CA, USA) in Tx-TBS with 1% NHS. Following rinsing in TBS, sections were incubated in biotinylated horse anti-rabbit antibody secondary, 1:200, for 3 h. The antibody was visualised using a standard ABC kit (Vectastain Elite, Vector Laboratories, Burlingame CA, USA) and the chromogen, 3-3′-diaminobenzadine (DAB kit, Vector Laboratories). Tissue sections were mounted onto gelatine-coated slides, dehydrated in successive concentrations of alcohol and coverslipped with DPX.

Cell bodies were counted under a Leica DM/RBE light microscope under x10 optical magnification in the ventral tegmental area (VTA) and the substantia nigra pars compacta (SNc) in one section at the level of the medial terminal nucleus of the accessory nucleus of the optic tract [37]. The density of TH-immunoreactive (TH-ir) terminals in the dorsal and ventral striatum was measured using ImageJ software (Version 1.42, National Institutes of Health, USA) from images of every 4th section taken with Leica DFC420 camera and Leica application V3.6 software. The camera was mounted on a free standing microscope (Wild-Heerburgg M420, so that the whole section could be visualised in the photograph. Optical density was measurement at 3 levels throughout the striatum, before their division into dorsal and ventral regions. The levels analysed relative to bregma were +0.12 mm, +0.5 mm, and +1.34 mm. Anatomical landmarks used to divide the striatum into dorsal and ventral parts were made by drawing a horizontal line across the striatum using the most dorsal point of the lateral ventricle for the first two sections and the anterior commissure for the latter.

*2.6. Statistical analysis*

All data are expressed as mean ± standard error of the mean (s.e.m.) and were analysed using the Statistical Software Package for the Social Science (SPSS v16). A significance level of $\alpha = 0.05$ was chosen for all comparisons. All behavioural data were analysed using univariate analysis of variance and Dunnett's post hoc test using the control group as reference. When appropriate, a bias score was calculated as (100× (ipsilateral paw or side)/contralateral paw or side). Behavioural results were used for bivariate correlation analysis with the histological measures of cell loss using Pearson correlation coefficients (one-tailed). Because of the presence of co-correlation between

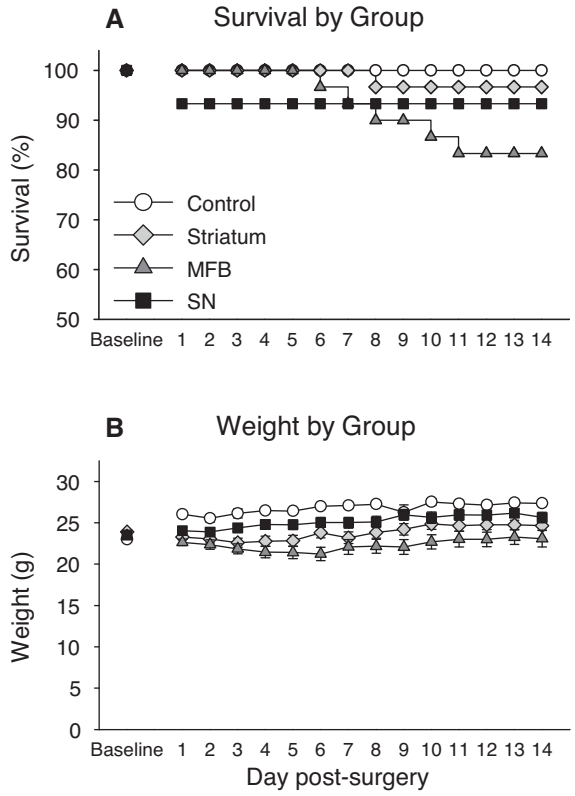


**Fig. 2.** Survival (A) and body weight (B) for the 14 consecutive days post lesion plotted per group. Baseline day was at the day of surgery. Note that intensive post-operative care was implemented for the 14 days post lesion. Free access to high caloric food (wet mash) was provided to all cages.

TH-ir cell loss in the SNc, in the VTA, and behavioural performance, we used partial correlation analysis to explore individual associations between behavioural performance and the respective areas of cell loss. Partial correlation analysis allows for controlling for the variance that is shared between two variables, allowing the contribution of variance associated with each variable to the overall variation to be assessed. Accordingly, co-correlation between SNc and VTA depletion was taken into account using partial correlation analysis for each of the behavioural assessments.

## 3. Results

### 3.1. Mortality rates and survival

The majority of mice (88%) recovered from the three lesion surgeries after 14 days of post-operative care (Fig. 2A). The mortality rates were low, with 83.3% of our MFB lesion mice surviving the surgery compared to up to 82% mortality that has been reported elsewhere [10,13]. The recovery rates observed in the present study were comparable with Francardo et al. [17] who report a survival rate of 80–100% over three experiments. In the present study, mice died or had to be sacrificed within the first 10 days post lesion only (Fig. 1A). However, in the majority of mice, weight loss and dehydration could be prevented by the feeding regime in combination with glucose-saline injections (Fig. 2B). The low mortality rate in the present study may have been the result of less severe dopaminergic depletion as compared to previous work. However, we have found that by implementing an intensive care protocol [17,38] mortality rates remained low. As recently described in Ref. [17] the intensive care protocol led to 100% survival in one experiment with bigger lesion effects than in the present study. Final group numbers for behavioural and histological assessment were Control mice: $n = 15$, striatum lesion mice: $n = 29$, MFB lesion mice: $n = 25$, and SN lesion mice: $n = 27$ (from an original group number of 30 per lesion group).

### 3.2. Histology

Representative examples of successful lesions are shown in Fig. 3 for each of the three lesion types respectively. All three groups exhibited clear loss of TH-ir cells in the substantia nigra on the side of the lesion and clear loss of TH terminal staining in the forebrain, most noticeably in the neostriatum. Extensive depletion was also seen in ventral striatal areas of the nucleus accumbens and olfactory tubercle in the MFB lesion group (Fig. 3C), which was also present but to a less marked degree in the SN and striatal lesion groups (Fig. 3B and D).

#### 3.2.1. Tyrosine hydroxylase immunoreactive cell counts

Overall, 6-OHDA administration caused a reduction of the numbers of TH-immunoreactive cell bodies in the SNc (Group, $F_{3,90} = 13.33$, $p < 0.001$) on the lesion side. The lesions caused a significant depletion of TH positive cells in the SNc of each of the three lesion groups, differing significantly from control (Fig. 4; striatum, $p < 0.001$; MFB, $p < 0.001$; SN, $p < 0.01$). The striatal and MFB lesion groups also had significant reductions in the number of TH-ir cells in the VTA (striatum, $p < 0.01$ and MFB, $p < 0.001$). However, there was no significant reduction in the percentage of TH-ir cells in the VTA in the SN lesion group, suggesting that lesions of the SN were more focal to the more laterally located cells of the ventral midbrain. Within each group the extent of lesions varied between relatively small to almost complete (remaining cells of contralateral: MFB: 1.33% to 109%; SN: 1.56% to 117.65%; striatum: 1.43% to 110.20%). As can be seen in Fig. 4 A, 20–50% of the mice in all groups exhibited relatively complete lesions (>90% depletion), whilst a number of mice in both the MFB and SN lesion groups had very small lesions (<25% depletion). The lesion success was highest in the striatum lesion group where the majority of mice exhibited at least partial lesions. The variability of the lesions is considered further as a covariate against behavioural deficits (Section 3.4, below).

#### 3.2.2. TH-ir cells correlate with TH-denervation of the striatum

The 6-OHDA lesion caused a reduction in TH-fibre density in the striatum on the side of the lesion (Group, $F_{3,89} = 17.57$, $p < 0.001$). All three lesion groups exhibited a reduced density in TH-ir fibres in the whole striatum that significantly differed from the control group (all, $p < 0.001$). The MFB lesion produced the largest reduction of overall striatal TH-ir fibres, resulting in a 37% reduction in the density of the contralateral side, followed by the striatal lesion group which had 41% of the contralateral density remaining and lastly the SN lesion resulted in 46% of contralateral density remaining. Individual analyses of dorsal striatal and ventral striatal density (Fig. 4C and D) revealed a significant effect of Group for the dorsal and ventral striatum (Group, $F_{3,89} = 18.72$ and 12.80, respectively, both $p < 0.001$). The density in the dorsal striatum differed significantly from control for all three lesion groups (all, $p < 0.001$). The ventral striatal density was also significantly reduced (Group, $F_{3,89} = 12.80$, $p < 0.001$), although to a lesser extent than dorsal striatal density. Post hoc analysis showed that all three types of lesions also significantly reduced ventral striatal density compared to the contralateral side (all, $p < 0.001$).

### 3.3. Behaviour

Data from the behavioural tasks are plotted in Fig. 5A–L and the results of the statistical analyses are presented in Table 1.

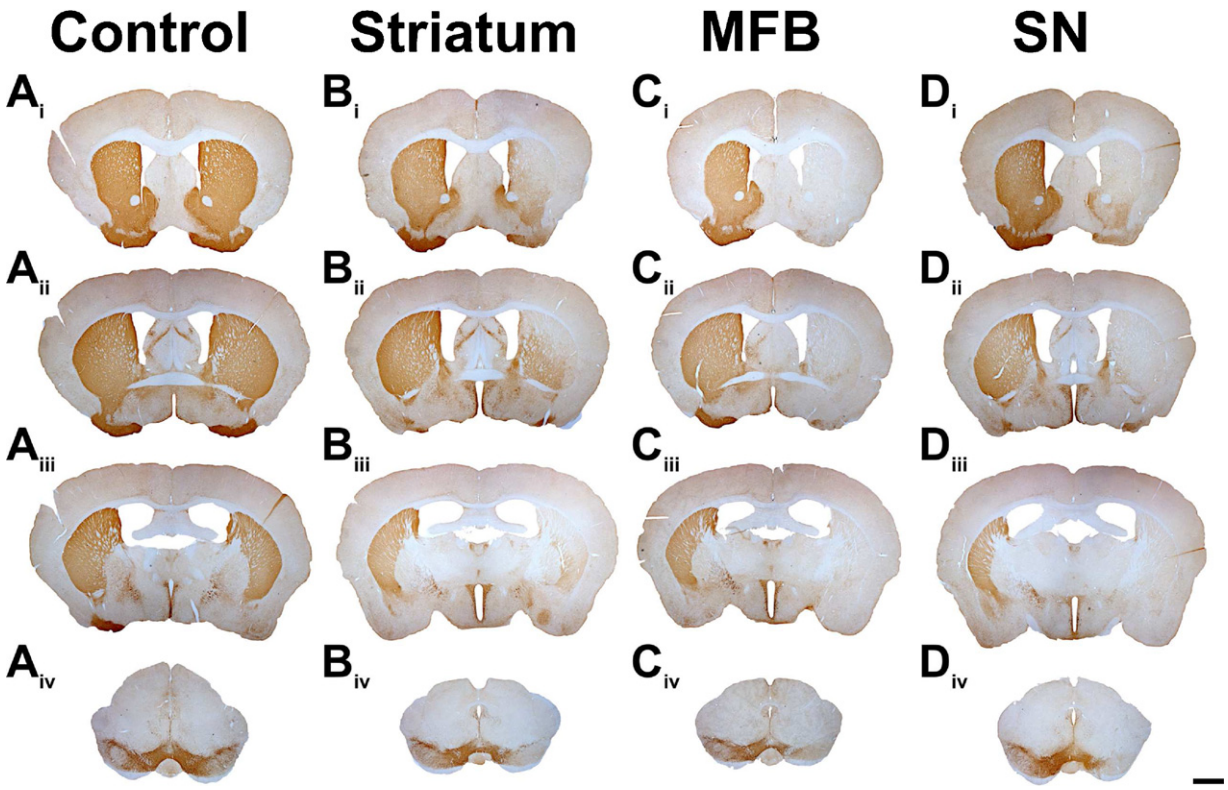


**Fig. 3.** Bright field photomicrographs of representative sections for successful lesions in coronal brain sections stained for TH-ir cells for each experimental group (A–D). Sections were taken approximately at levels (i) +1.0 mm, (ii) −.2 mm, (iii) −1.3 mm, and (iv) −5.2 mm from Bregma. Scale bar represents 1 mm. Note the lack of TH-ir staining one the side ipsilateral to the lesion (B–D). Dopaminergic cell bodies are relatively spared in the VTA region of the striatal (B) and the SN (D) lesion group companied with TH-ir fibres in the ventral striatum.

### 3.3.1. Staircase test

Control mice retrieved the same number of pellets from both sides of the staircase test and no impairments were observed in any of the lesion groups (Fig. 5A; Group, $F_{3,91} = 0.58$, n.s.).

### 3.3.2. Corridor test

Control mice spent similar amounts of time retrieving pellets from the left and right side of the corridor. However, mice with MFB or striatum lesions demonstrated a significant ipsilateral preference in collecting pellets compared to the control group (Fig. 5B; Group, $F_{3,91} = 4.62$, $p < 0.01$, post hoc: both, $p < 0.01$) whilst the SN lesion group did not display a significant side preference.

### 3.3.3. Balance beam

Although the group effect on the balance beam failed to reach conventional levels of significance (Fig. 5C; Group, $F_{3,90} = 2.65$, $p = 0.054$), the MFB and striatum lesion groups show a clear trend for increased foot slips on the side contralateral to the lesion, when each of the lesion groups is compared to the control group individually (both, $p < 0.05$).

### 3.3.4. Cylinder test

The cylinder test revealed significant group differences in paw use preference for eliciting weight-bearing touches (Fig. 5D; Group, $F_{3,91} = 4.25$, $p < 0.01$). The striatum and MFB lesion groups predominantly use the paw ipsilateral to the lesion in exploration of the cylinder walls, compared to control mice which use both paws equally ($p < 0.01$ and $p < 0.05$, respectively).

**Table 1**
Correlations between each behavioural test and SNc cell counts (% of contralateral).

| Test | Total | Control | Striatum | MFB | SN |
|---|---|---|---|---|---|
| Staircase (%) | −0.076 | 0.213 | −0.202 | −0.009 | −0.015 |
| Corridor (%) | 0.557** | 0.030 | 0.317 | 0.529** | 0.734** |
| Balance beam (%) | −0.399** | −0.097 | −0.104 | −0.283 | −0.778** |
| Cylinder (%) | −0.581** | −0.416 | −0.336* | −0.751** | −0.442* |
| Rotarod (s) | 0.304** | 0.198 | 0.036 | 0.215 | 0.309 |
| Stepping bias (%) | −0.254** | 0.358 | 0.223 | −0.205 | −0.269 |
| Gait (stride length left) | 0.184* | −0.049 | 0.087 | −0.009 | 0.248 |
| Cage lid (s) | 0.171 | 0.124 | 0.027 | −0.132 | 0.052 |
| Activity (beam breaks) | 0.136 | 0.003 | −0.009 | 0.258 | 0.110 |
| Apomorphine rotation (%) | 0.548** | −0.259 | 0.359* | 0.665* | 0.476** |
| Amphetamine rotation (%) | −0.698** | −0.193 | −0.623** | −0.810** | −0.619** |
| Spontaneous rotation (%) | −0.485** | 0.003 | −0.153 | −0.671** | −0.630** |

Pearson correlation coefficients ($r$).
* $p < 0.05$.
** $p < 0.01$.

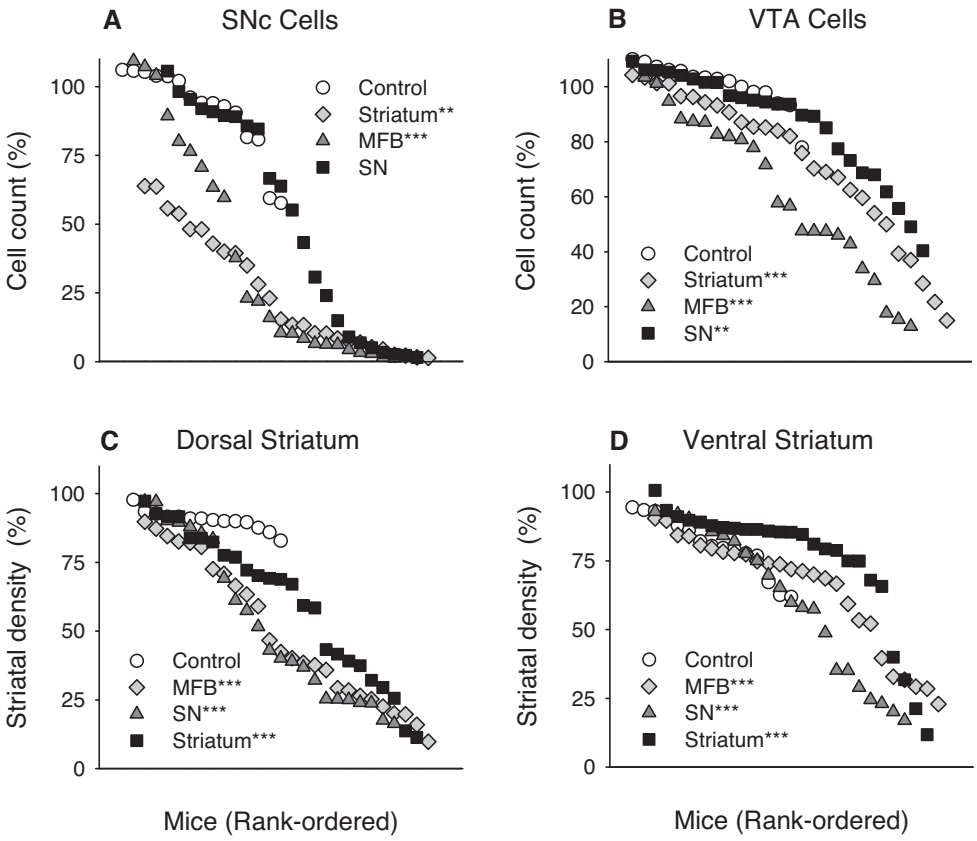


**Fig. 4.** Cell counts in the ventral mesencephalon and striatal TH-ir fibre density rank-ordered per group. Ventral mesencephalic cell counts expressed as percent of contralateral for SNc (A) and VTA (B). Striatal density measures of TH-ir fibres are expressed as percent of contralateral for the dorsal (C) and ventral (D) striatum, respectively. Number of asterisks denotes different from control group (Dunnett's post hoc) at the <0.05, the <0.01, and the <0.001 level of significance.

*3.3.5. Rotarod*

The latency to fall from the rotating rod was significantly affected by the lesion (Fig. 5E; Group, $F_{3,91} = 3.76$, $p < 0.05$). All three lesion groups showed a shorter latency to stay on the accelerating version of the rotarod compared to control (striatum, $p < 0.05$; MFB, $p < 0.01$; SN, $p < 0.05$).

*3.3.6. Stepping test*

The stepping test revealed a difference in the number of steps the mice made (Group, $F_{3,88} = 4.50$, $p < 0.01$), with control mice making $8.12 \pm 0.51$ adjusting steps compared to the striatum lesion group $6.46 \pm 0.36$ adjusting steps, the MFB lesion group $6.51 \pm 0.37$ adjusting steps and the SN lesion group with $7.77 \pm 0.36$ adjusting steps. Dunnett's post hoc test showed that both the striatum and the MFB lesion group made significantly fewer adjusting steps compared to mice of the control group (both, $p < 0.05$). However, the deficit in the MFB and striatal lesions groups was bilateral, rather than affecting primarily the contralateral side, so that there was no significant group difference in the side bias index of adjusting steps made on the stepping test (Fig. 5F; Group, $F_{3,85} = 1.63$, n.s.).

*3.3.7. Gait analysis*

There was no difference between the groups in stride length as measured using the paw print method (Fig. 5G; Group, $F_{3,81} = 0.71$, n.s.).

*3.3.8. Inverted cage lid*

There was a significant reduction in the time lesion mice were able to cling onto the inverted cage lid (Fig. 5H; Group, $F_{3,91} = 3.59$, $p < 0.05$). Post hoc analysis revealed a difference between the striatum lesion group and the control group on this measure of grip strength ($p < 0.05$), with the striatum lesion group spending less time on the inverted grid.

*3.3.9. Locomotor activity*

No difference between the experimental groups could be detected using the locomotor activity test (Fig. 5I; Group, $6_{3,91} = 1.51$, n.s.)

*3.3.10. Rotation*

Assessment of spontaneous rotation in a cylinder revealed significant differences between the groups (Fig. 5J; Group, $F_{3,90} = 10.597$, $p < 0.001$). Post hoc tests demonstrated that only the MFB lesion group displayed a significant spontaneous rotational bias towards the side of the lesion ($p < 0.01$) compared to control. Methamphetamine-induced rotation revealed differences in the directional bias of rotation between the groups (Fig. 5K; Group, $F_{3,91} = 4.33$, $p < 0.01$). The striatum and the MFB lesion groups rotated significantly towards the side of the lesion when compared to mice of the control group (both, $p < 0.01$; MFB, $p < 0.01$). There was a significant group difference when mice were challenged with apomorphine (Fig. 5L; Group, $F_{3,89} = 6.09$, $p < 0.01$). Post hoc testing showed that mice of the striatum lesion group ($p < 0.05$) and mice of the MFB lesion group ($p < 0.01$) displayed significantly more contralateral rotations when compared to the control group.

*3.3.11. Summary of behavioural tests*

Of all the tests administered, eight tests were found to be sensitive to dopamine-depleting lesions of the nigrostriatal pathway: the corridor test, the cylinder test, the rotarod test, the balance beam test, the inverted cage lid test and all three types of rotational assessments. However, because of the large variation in lesion size,

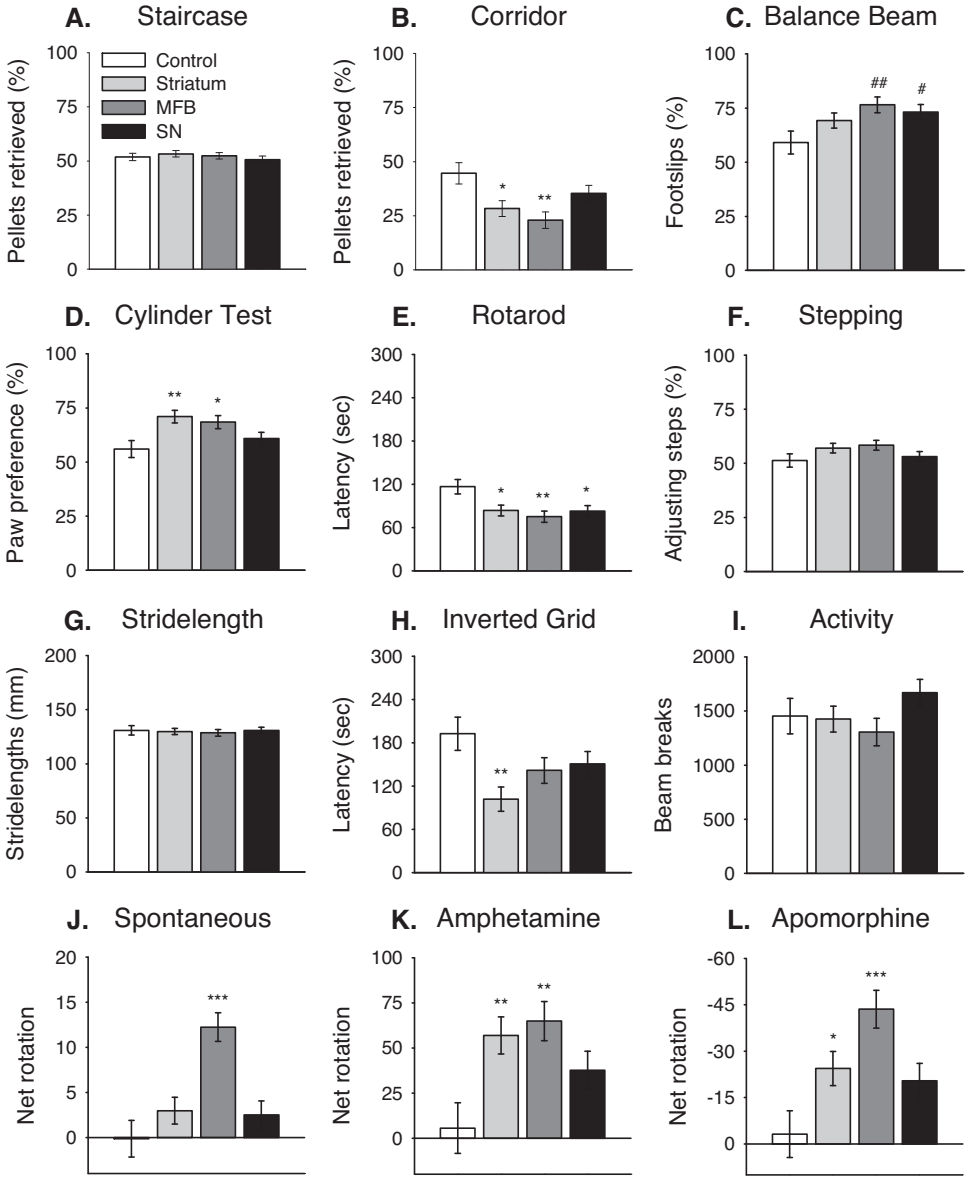


**Fig. 5.** Lesion effects on behavioural tasks. The effects of lesions on each of the behavioural tests administered. All percentages are expressed as percent of contralateral. Number of asterisks denote significant different from control (Dunnett's post hoc) at the <0.05, <0.01, and <0.001 level of significance, respectively. Number of hashes denote significant different from control (Dunnett's post hoc) at the $p < 0.05$ and 0.01 level, although ANOVA did not return significant at the 0.05 level.

some of the behavioural effects of the lesions on group performance might be overshadowed by the mice with undetectable, small or ineffective lesions. A subset of well-lesioned mice, as defined by three criteria (amphetamine-induced rotation >5 turns/min, >65% forelimb preference on the cylinder test and less than 75 s latency to fall on the rotarod), was taken for further investigation in Ref. [39]. It can be seen that analysis restricted to a subset of mice with robust lesions yields a similar profile of behavioural change and differences between the three lesion conditions, albeit with lower within-group variance to that provided in the present analyses in the full (unselected) groups.

### 3.4. Correlational associations between behaviour and histology

#### 3.4.1. Dopaminergic cell count in the SNc correlates with behavioural impairment

The proportion of dopaminergic cell bodies remaining in the ipsilateral SNc (Table 1; Fig. 6) correlated well with contralateral performance on the amphetamine-induced rotation test, the cylinder test, and the corridor test ($r > 0.50$, all $p < 0.01$). Significant correlations were also evident for the spontaneous rotation test, the balance beam, the apomorphine-induced rotation test and the rotarod test (all $0.50 > r > 0.30$, $p < 0.01$). Although correlations for the stepping test ($p < 0.01$) and the gait analysis ($p < 0.05$) were also significant, the effect sizes on these two tests were relatively small (all $r < 0.30$). The remaining three tests (staircase, inverted cage lid, and locomotor activity) did not correlate well with the percentage of TH-ir cells in the SNc.

When exploring the correlations between the proportion of remaining SNc cells and performance on the behavioural tests for each of the three lesion groups individually, the amphetamine-induced rotation test was found to produce the most robust correlation effects with all three lesion groups (all, $p < 0.01$). The other test that correlated significantly with all three lesion groups was the cylinder test (all, $p < 0.05$). Furthermore, these two tests along with apomorphine-induced rotations provided the only measures that correlated with the percentage of remaining dopaminergic nigral cells in the striatal lesion group. SNc cell counts

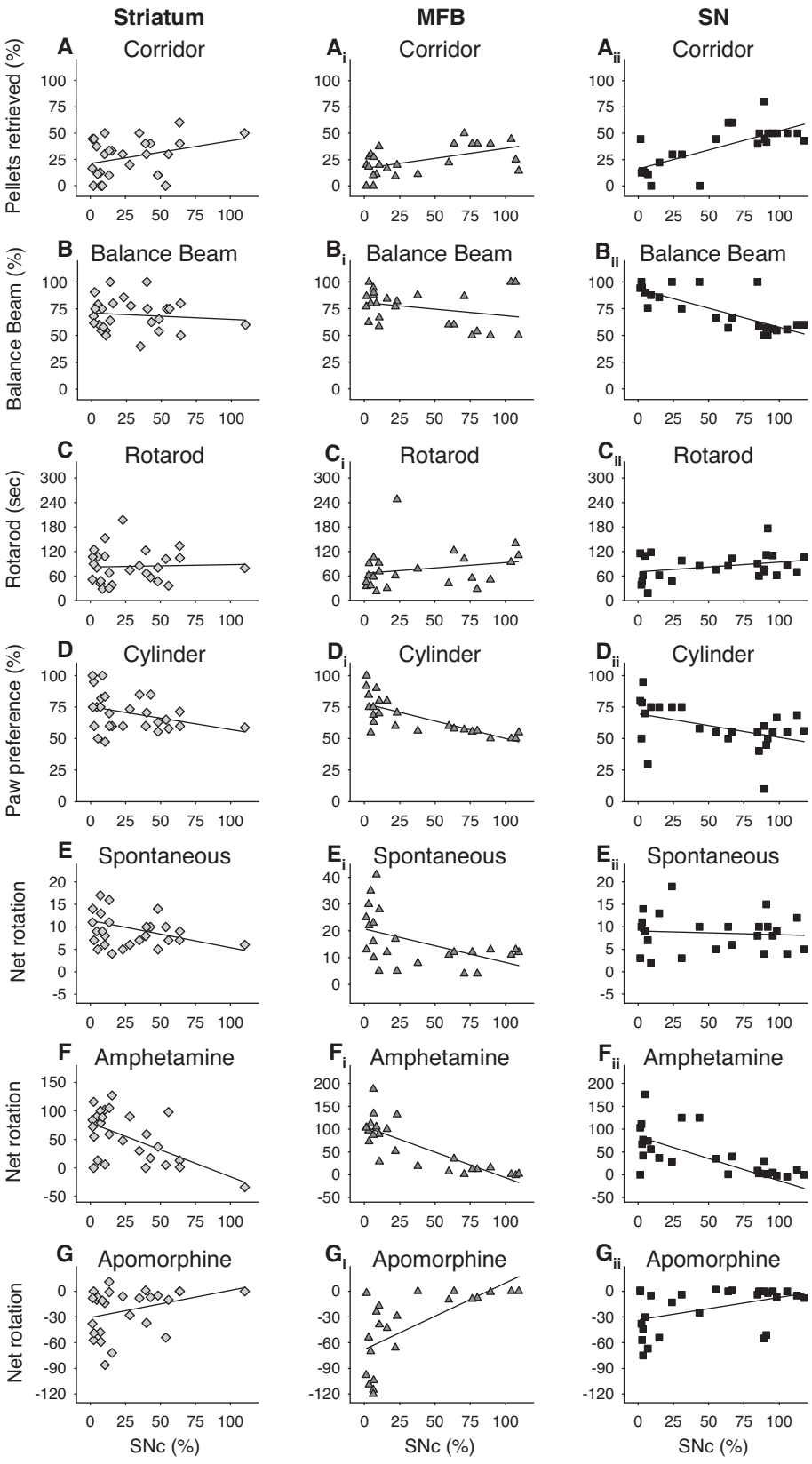


**Fig. 6.** Correlations between selected behavioural tests and SNc cell counts in respective lesion groups, where striatal (A–F), MFB (Ai–Fi) and SN groups (Aii–Fii). All percentages are expressed as % of contralateral.

**Table 2**
Correlations between each behavioural test and VTA cell counts (% of contralateral).

| Test | Total | Control | Striatum | MFB | SN |
|---|---|---|---|---|---|
| Staircase (%) | 0.016 | −0.201 | −0.234 | 0.213 | 0.317 |
| Corridor (%) | 0.383** | −0.252 | −0.035 | 0.568** | 0.532** |
| Balance beam (%) | −0.425** | −0.331 | −0.322 | −0.408* | −0.653** |
| Cylinder (%) | −0.516** | −0.599** | −0.340* | −0.797** | −0.180 |
| Rotarod (s) | 0.275** | 0.233 | 0.018 | 0.296 | 0.171 |
| Stepping bias (%) | 0.400** | 0.054 | 0.374* | 0.333 | 0.209 |
| Gait (stride length left) | 0.081 | −0.189 | −0.063 | 0.297 | 0.151 |
| Cage lid (s) | 0.080 | 0.343 | −0.325* | −0.254 | 0.368* |
| Activity (beam breaks) | 0.156 | −0.222 | −0.019 | 0.185 | 0.252 |
| Apomorphine rotation (%) | 0.450** | −0.286 | 0.355* | 0.313 | 0.422* |
| Amphetamine rotation (%) | −0.497** | 0.232 | −0.460** | −0.483** | −0.416* |
| Spontaneous rotation (%) | −0.497** | −0.040 | −0.270 | −0.530** | −0.239 |

Pearson correlation coefficients ($r$).
* $p < 0.05$.
** $p < 0.01$.

**Table 3**
Correlations and partial correlations between each behavioural test and cell counts (% of contralateral), calculated for the animals of the 3 lesioned groups only.

| Test | Total SNc | Total VTA | Partial SNc (controlling for VTA) | Partial VTA (controlling for SNc) |
|---|---|---|---|---|
| Staircase (%) | −0.095 | 0.036 | −0.139 | 0.108 |
| Corridor (%) | **0.570*** | 0.372** | **0.471**** | 0.078 |
| Balance beam (%) | −0.388** | **−0.422**** | −0.202* | **−0.268*** |
| Cylinder (%) | **−0.554**** | −0.473** | **−0.396**** | −0.237* |
| Rotarod (s) | 0.182 | 0.181 | 0.099 | 0.097 |
| Stepping bias (%) | **−0.219*** | −0.214* | −0.122 | −0.113 |
| Gait (stride length left) | 0.138 | 0.034 | 0.144 | −0.053 |
| Cage lid (s) | 0.056 | −0.037 | 0.092 | −0.083 |
| Activity (beam breaks) | 0.170 | 0.188 | 0.080 | 0.113 |
| Apomorphine rotation (%) | **−0.322**** | −0.302** | −0.193 | −0.156 |
| Amphetamine rotation (%) | **−0.681**** | −0.455** | **−0.577**** | −0.123 |
| Spontaneous rotation (%) | −0.441** | **−0.456**** | −0.252* | **−0.280**** |

Bold numbers highlight the larger of the two significant correlations of each test against the SNc or VTA cell counts for the overall correlation pair and the partial correlation pair, respectively.
Pearson correlation coefficients ($r$).
* $p < 0.05$.
** $p < 0.01$.

in the MFB and the SN lesion groups also correlated significantly with performances on the spontaneous rotation test and the corridor test (both, $p < 0.01$). The balance beam test only correlated with the proportion of remaining cells in the SN lesion group ($p < 0.01$). No other test correlated significantly with cell loss in the lesion groups.

### *3.4.2. Dopaminergic cell counts in the VTA correlate with behavioural performance*

The proportion of remaining cells in the VTA for all groups correlated with performance on the cylinder, balance beam, stepping and corridor tests, the amphetamine-induced, spontaneous and apomorphine-induced rotation and performance on the rotarod apparatus (all, $p < 0.01$, Table 2). When assessing each groups' performance individually, the proportion of remaining VTA cells correlated significantly with amphetamine-induced rotation in all three lesion groups (striatum and MFB, both $p < 0.01$; SN, $p < 0.05$). Correlations between VTA cells and performance on the corridor test and balance beam were evident for the MFB and SN lesion groups (both $p < 0.05$), whilst the inverted cage lid test and the apomorphine-induced rotations correlated with the striatum and SN lesion groups (all, $p < 0.05$). The cylinder test correlated significantly with the striatum and the MFB lesion group (striatum, $p < 0.05$; MFB, $p < 0.01$). The stepping test only correlated with the

**Table 4**
Correlations between each behavioural test and dorsal striatal TH-ir density (% of contralateral).

| Test | Total | Control | Striatum | MFB | SN |
|---|---|---|---|---|---|
| Staircase (%) | −.129 | −.231 | −.278 | .159 | −.219 |
| Corridor (%) | .291** | −.480* | −.033 | .634** | .178 |
| Balance beam (%) | −.272** | .157 | −.172 | −.217 | −.278 |
| Cylinder (%) | −.301** | −.008 | −.039 | −.587** | .038 |
| Rotarod (s) | .386** | .553* | −.126 | .594** | .283 |
| Stepping bias (%) | −.179 | .205 | .183 | −.213 | −.251 |
| Gait (stride length left) | .294** | −.048 | .200 | .415* | .400* |
| Cage lid (s) | .167 | .197 | .299 | −.457* | .116 |
| Activity (beam breaks) | .012 | .003 | .029 | .175 | −.065 |
| Apomorphine rotation (%) | −.211* | .100 | .180 | −.366 | .071 |
| Amphetamine rotation (%) | −.380** | .327 | −.324* | −.513** | −.083 |
| Spontaneous rotation (%) | −.371** | −.483* | .040 | −.526** | −.509** |

Pearson correlation coefficients ($r$).
* $p < 0.05$.
** $p < 0.01$.

**Table 5**
Correlations between each behavioural test and ventral striatal TH-ir density (% of contralateral).

| Test | Total | Control | Striatum | MFB | SN |
|---|---|---|---|---|---|
| Staircase (%) | −.125 | −.007 | −.408* | .141 | −.093 |
| Corridor (%) | .218* | −.203 | −.308 | .590** | .034 |
| Balance beam (%) | −.247* | .472 | −.221 | −.182 | −.153 |
| Cylinder (%) | −.221* | .134 | .126 | −.498** | .088 |
| Rotarod (s) | .289* | .428 | −.267 | .571** | .001 |
| Stepping bias (%) | −.180 | .135 | .086 | −.266 | .060 |
| Gait (stride length left) | .324** | −.035 | .192 | .320 | .575** |
| Cage lid (s) | .181* | .204 | .098 | −.363* | .399* |
| Activity (beam breaks) | .032 | −.584* | −.119 | .129 | .101 |
| Apomorphine rotation (%) | −.291** | −.096 | −.092 | −.417* | .022 |
| Amphetamine rotation (%) | −.293** | .250 | −.062 | −.441* | .007 |
| Spontaneous rotation (%) | −.355** | −.467* | .102 | −.409* | −.109 |

Pearson correlation coefficients ($r$).
* $p < 0.05$.
** $p < 0.01$.

striatum lesion group ($p < 0.05$) and the spontaneous rotation test only correlated with the MFB lesion group ($p < 0.01$) and the respective VTA cell counts.

### 3.4.3. Partial correlations suggest distinct contributions of SNc and VTA to the different behaviours deficits

We explored which of the tests are particularly sensitive to depletion in either of the two ventral midbrain dopaminergic nuclei using a partial correlations analysis, by correlating behavioural performance over all lesioned groups (excluding control group mice) with nigral cell counts taking into account the influence of VTA cell counts with partial correlations, and vice versa. As can be seen in Table 3, seven of the behavioural tests correlated well with cell loss in both the SNC and VTA. However, cell loss in the VTA itself correlates highly with cell loss in the nigra ($r = 0.630$, $p < 0.001$). Of the 7 tests, which correlate with VM cell loss, only the balance beam test and spontaneous rotation correlated higher with VTA cell counts than with SNc cell counts. After accounting for the influence of the two dopaminergic nuclei, three interesting results emerged. Firstly, removing the influence of SNc cell counts eliminated the significance of several behavioural tests correlating with the VTA, including the corridor and cylinder tests and both tests of drug-induced rotation, leading to the assumption that behavioural performance of the tests is mainly driven by SNc cell counts. Secondly, we found that the two tests – balance beam and spontaneous rotations – that had higher overall correlations between VTA cell counts and behavioural performance did remain correlated higher, even after performing co-correlation with SNc cell counts. Interestingly this points towards a specific influence of the VTA on those behavioural tests. Thirdly, the results demonstrated that apomorphine-induced rotation and the stepping bias were no longer correlated with cell loss in either the SNc or VTA in isolation, and therefore it can be concluded that both structures need to be depleted to produce a significant deficit under the present circumstances.

### 3.4.4. Behavioural performance correlates with striatal density measures

Correlations between striatal density measures and behavioural tests are shown in Tables 4 and 5, for dorsal striatum and ventral striatum, respectively. Dorsal striatal density correlated highest with the MFB lesion group on the corridor test, the cylinder test, the rotarod test, the amphetamine and the spontaneous rotation (all $r > 0.500$; $p < 0.01$), with gait analysis ($r = 0.415$; $p < 0.05$) and the cagelid test ($r = -0.457$; $p < 0.05$). Dorsal striatal TH-ir density also correlated with the SN lesion group on the gait analysis ($r = 0.400$; $p < 0.05$) and the spontaneous rotation test ($r = -0.509$; $p < 0.01$). The striatal lesion group only correlated with dorsal striatal TH-ir density on the amphetamine induced rotation test ($r = -0.324$; $p < 0.05$). TH-ir density in the ventral striatum also correlated

**Table 6**
Behavioural outcome measures in a subset of mice that were selected on the basis of < 20% SNc cells remaining and the animals of the untreated control group.

| Test | $F_{3,45}$ | Control | Striatum | MFB | SN |
|---|---|---|---|---|---|
| SNc (%) | 276.473*** | 7.37 ± 1.25 | 7.37 ± 1.25*** | 6.51 ± 1.24*** | 5.73 ± 1.58*** |
| VTA (%) | 21.381*** | 57.65 ± 7.28 | 57.65 ± 7.28*** | 42.61 ± 6.19*** | 69.40 ± 6.17** |
| Dorsal density | 48.017*** | 20.47 ± 6.98 | 20.47 ± 6.98*** | 20.49 ± 6.17*** | 17.25 ± 8.06*** |
| Ventral density | 18.759*** | 48.43 ± 9.64 | 48.43 ± 9.64*** | 28.73 ± 7.86*** | 40.70 ± 7.99*** |
| Staircase (%) | 0.501 | 54.30 ± 2.07 | 54.30 ± 2.07 | 52.07 ± 2.04 | 50.34 ± 3.62 |
| Corridor (%) | 7.117** | 23.10 ± 4.79 | 23.10 ± 4.79** | 18.21 ± 3.34** | 16.42 ± 4.55** |
| Balance beam (%) | 5.323** | 69.27 ± 3.91 | 69.27 ± 3.91 | 80.63 ± 3.67** | 89.74 ± 2.95** |
| Cylinder (%) | 6.204** | 73.75 ± 4.59 | 73.75 ± 4.59** | 77.77 ± 3.68*** | 69.12 ± 7.18 |
| Rotarod (s) | 7.35*** | 76.96 ± 10.47 | 76.96 ± 10.47** | 58.83 ± 7.74*** | 71.44 ± 13.59** |
| Stepping bias (%) | 1.304 | 57.81 ± 1.88 | 57.81 ± 1.88 | 61.76 ± 6.95 | 58.28 ± 6.34 |
| Gait (stride length left) | 0.878 | 128.81 ± 3.66 | 128.81 ± 3.66 | 122.58 ± 3.65 | 124.06 ± 6.09 |
| Cage lid (s) | 1.923 | 109.86 ± 19.07 | 109.86 ± 19.07** | 151.53 ± 30.71 | 124.21 ± 42.73 |
| Activity (beam breaks) | 0.959 | 1288.79 ± 127.16 | 1288.79 ± 127.16 | 1133.83 ± 112.52 | 1479.38 ± 348.19 |
| Apomorphine rotation | 10.855*** | −32.07 ± 8.02 | −32.07 ± 8.02* | −66.25 ± 12.10*** | −40.63 ± 9.86** |
| Amphetamine rotation | 20.660*** | 78.36 ± 9.46 | 78.36 ± 9.46*** | 101.75 ± 10.70*** | 87.62 ± 15.17*** |
| Spontaneous rotation | 16.351*** | 4.21 ± 2.39 | 4.21 ± 2.39 | 20.33 ± 3.37*** | 6.12 ± 1.67 |

All data represent mean ± s.e.m.
Asterisks in the first column ($F$-ratio) indicate a significant main effect whereas asterisks for the group columns (striatum, MFB, SN) denote significant differences from control.
* Significance for $p < 0.05$.
** Significance for $p < 0.01$.
*** Significance for $p < 0.001$.

highest with behavioural tests in the MFB lesion group on the corridor test and the rotarod test (all $r > 0.500$; $p < 0.01$). Strong correlations were also found in the cylinder test, the cagelid test, and all three types of rotation tests (all $0.50 > r < 0.30$; $p < 0.05$). In the SN lesion group, ventral striatal TH-ir density correlated with the gait analysis ($r = .575$; $p < 0.01$) and the cagelid test ($r = 0.399$; $p < 0.05$) and in the striatal lesion group only the performance on the staircase test correlated significantly with ventral striatal density ($r = -.408$, $p < 0.05$).

### 3.5. Behavioural performance of a subset of well-lesioned mice

Lesions in mice have shown to be highly variable in terms of lesion extent and behavioural performance. Because of this variability we have chosen to select a subset of mice with <20% SNc cells left on the lesioned side and compared these new re-grouped animals with all 15 mice of the control group. As can be seen in Table 6 in these subsets of mice, there were significant behavioural differences in all three lesion groups in performance on the corridor test, the rotarod test, amphetamine-induced and apomorphine-induced rotation test. The balance beam teased out a significant difference between the control group and two of the lesion groups (MFB and SN), whereas the cylinder test showed significant differences between the striatal lesion group and the MFB lesion group with the control group. When comparing the new, restricted dataset, with the original analysis (which included all mice), all the *F*-ratios increased leading to smaller *p*-values in all tests that were significant in the previous, unrestricted analysis. Furthermore, performance on the balance beam was now significant at conventional levels of significance (previously $p = 0.052$) and two of the lesion groups differed from control (MFB and SN). The SN lesion group as a whole did not perform differently from the control group in the original analyses of drug-induced rotations, but after restricting the analysis of mice to those with severe TH-ir cell depletion, the SN group performance was significantly different to control mice on the amphetamine- ($p < 0.001$) and apomorphine-induced ($p < 0.01$) rotation tests. All other behavioural measures were similar to the original analysis.

## 4. Discussion

Our study sought to provide a detailed comparison of unilateral lesions to the nigrostriatal dopamine pathway at three different sites in mice, including behavioural assessment and correlation between motor function and pathological changes in the striatum and ventral mesencephalon. A primary aim was to identify tests, which are appropriate in each model for the identification of an effective lesion, allowing the selection of well-lesioned mice to be carried forwards into other studies.

### 4.1. Lesion efficiency and survival/mortality

All lesions produced a marked degeneration of the dopaminergic neurons in the SNc and a subsequent denervation of TH-ir fibres in the striatum. As expected, due to the compactness of the bundle, the lesions aimed at the MFB were more complete, extending their degeneration to parts of the VTA and, therefore, to a large proportion of the ventral striatum. The degree of degeneration and denervation in the VTA and ventral striatum was more variable in the other two lesion groups, with dopamine depletion primarily restricted to the SNc and the dorsal striatum in the majority of cases. As can be seen in Fig. 4, lesion success has been highly variable in the present study with only a subset of mice displaying substantial cell depletion in the SNc and VTA and reduction in striatal TH-ir density. This variability explains why not all tests that correlate with SNc depletion show significant group differences as well.

Previous studies have advocated use of the SN and striatum lesion models mainly because of the high mortality rate that has been reported with lesions aimed at the MFB [10,13,38]. Recently it has been reported that implementing intensive post-operative care can reduce mortality to ethically acceptable levels [10,12,38,39]. In support of this, despite initial reports of >80% mortality rates, a recent study reports survival rates of 100% with extensive DA depletion [17]. Although we report the highest mortality rate in the MFB lesion group, which produced the most extensive lesions, the percentage of mice lost was relatively very low, at 17.7%, in comparison with previous studies. Mortality rate and weight loss appears to be directly linked to the extent and time-course of dopaminergic depletion [24,40]. The more extensive MFB lesion mice were the most vulnerable. Moreover, the critical time window reflected the location of the lesion, with lesions of the striatum having a slower degeneration and thus longer post-surgical period during which survival might be compromised [12,13,41]. Severe spontaneous rotations were exhibited by those mice suffering significant weight loss, a behaviour which likely impacted on their ability to eat and drink.

### 4.2. Lesion-induced deficits on behavioural tasks

The tests that were most sensitive to distinguish mice of the respective lesion groups from untreated controls were the corridor test (MFB, striatum), the cylinder test (striatum), the balance beam (MFB, SN), the rotarod (all three groups), the inverted cage lid test (striatum), and the rotational assessments (Spontaneous, MFB; Amphetamine, all three groups; SN, striatum, MFB). Of all of these, the amphetamine rotation test was consistently the most reliable (see below).

The cylinder test, which is a standard tool used for identifying unilateral lesions in the rat model of PD, has also been seen to be sensitive to 6-OHDA lesions in mice – of the striatum in the present study, and of the SN in others [10,13,16]. The corridor test [20], which has been shown to be sensitive to unilateral 6-OHDA lesions aimed at the mouse SN [13], was sensitive to lesion-induced deficits in the present study. Interestingly, lesions aimed at the striatum and the MFB produced group effects different from control mice on this task, whereas lesions aimed at the SNc did not [13]. The differences between Grealish et al. [13] and the present study most likely results from selection of the experimental cohort and their respective overall degree of cell loss. Grealish et al. pre-selected mice after the lesion on the basis of a series of behavioural tests. Out of 122 lesion mice, which were tested behaviourally, 40 mice were selected based on their behavioural performance to represent subjects of a mild, intermediate and severe lesion type [13], where even mice with a 'mild' lesion had a mean reduction of TH-ir SNc cells of 71.4%. In our initial analysis we did not remove any mice from the lesion cohorts and hence incorporated a wider range of dopamine depletions and lesion deficits (See Figs. 4 and 2 in Ref. [16]). In that study, motor impairments on the rotational and corridor assessment are seen only in mice with >60% dopaminergic depletion. When we selected only severely lesioned mice post hoc we also found that group effects became stronger. In keeping with this, the largest effect of the lesion, in the unrestricted dataset, was seen when the site of injection was aimed at the neuronal terminals in the striatum, which was the group with the largest percentage of lesion mice with the smallest within-group variability, compared to the other two lesion groups.

Of particular interest are the results of the drug-induced rotations, as these tests have been the most commonly used in assessing success rate of unilateral lesions and cell replacement in the unilateral rat model [42]. In the reviewed mouse literature, a wide

range of doses of amphetamine and apomorphine are reported for the assessment of unilateral dopamine depletion in mice, e.g. amphetamine, from 0.25 to 8.0 mg/kg [13,15,16,43,44]. The doses of 2.5 mg/kg methamphetamine and 0.05 mg/kg for apomorphine used here were both found to induce circling behaviour without the development of significant stereotyped behaviour, which can pose a significant risk at the highest doses. The use of apomorphine as a measure of lesion success is slightly contentious; Grealish et al. [13] report a robust and reliable response at 0.1 mg/kg, whilst Iancu et al. [16] found apomorphine-induced rotation (0.5 mg/kg) and percentage of SNc cell loss poorly correlated. Critically apomorphine produces significant sensitisation of motor responding when administered repeatedly, and Grealish et al. report a reliable response following 3 administrations. Furthermore, in the present study apomorphine did not discriminate between SN lesion and control groups, most likely because this group had the highest percentage of cells remaining in the ventral mesencephalon, whereas, at least in rats, a denervation beyond 90% appears necessary to induce consistent supersensitivity and apomorphine rotation [45]. It is important to note that higher doses of apomorphine may induce stereotyped behaviour, which in turn diminishes the rotational response of the mice (which has been reported in Ref. [16]). Also the negative results on some of the behavioural tests confirm findings of others. For example, the stepping test in mice has not been found to be sensitive to unilateral 6-OHDA lesions of the SNc [13], although this test is sensitive to unilateral dopaminergic depletion in the rat [13,27,41]. Furthermore, the gait analysis/paw print test has been shown to be sensitive to abnormalities in gait of transgenic and lesioned rats and mice [32,46,47], but has not been sensitive enough to detect differences between lesion and control mice in the present study. The locomotor activity test, which did not show significant differences between any of the groups, was similarly uninformative in the Lundblad et al. [10] study, where a small effect was reported which was subject to spontaneous recovery after the first testing session. These results indicate that some of the behavioural screens used are not sensitive to small to medium sized lesion effects.

### 4.3. Behavioural performance correlates with ventral mesencephalic cell loss

6-OHDA injections aimed at the striatum produced a lesion in all cases over a smaller range of depletions (0–70% of contralateral), whereas the other two lesion groups produced more variable lesions over a wider range of depletions (e.g. 0–110% of contralateral, but see Fig. 5). It is worth noting that this difference in range of striatal TH depletion, as well as the variable destruction of VTA cells, may impact upon subsequent correlation analyses. Despite this, the statistical analyses clearly demonstrated that the three lesion groups differed in their relationship between TH cell loss and performance on many of the behavioural tests. Whilst performance on all tests of rotational behaviour (drug-induced and spontaneous-rotation and the cylinder test) correlated well with individual lesions, the corridor and balance beams tests also provided interesting means of discriminating lesion types. Interestingly, when correlating only the lesioned animals' cell counts with behavioural performance, it emerged that the majority of tests correlated higher with SNc rather than VTA cell counts.

Depletion of dopamine levels in the nucleus accumbens (NAcc), one of the main targets of the VTA, and dopamine levels in the striatum have differential effects on drug-induced rotation in the rat model of PD. After almost complete destruction of mesencephalic dopaminergic neurons by means of injection of 6-OHDA into the bundle, amphetamine-induced rotation could be observed in all rats [48]. Subsequent grafting of TH rich tissue into either the striatum only, or the striatum plus the NAcc, resulted in the expected decrease in rotational behaviour, but rats that received grafts in the NAcc only did not demonstrate a decrease in rotations, suggesting that dorsal striatal dopamine is critically involved in the rotational behaviour [48]. Furthermore, it has been demonstrated that destruction of the NAcc or the VTA alone is not sufficient to induce rotational behaviour in the rat, and indeed rats that receive both unilateral striatal and bilateral NAcc lesions rotate less than those with an intact NAcc [49,50]. Additionally, bilateral lesions to the NAcc, but not the striatum reduced locomotor activity, relative to sham rats, after treatment with low doses of amphetamine [51]. Thus, these studies suggest that during the amphetamine-induced rotation task, DA levels in the striatum determine the direction and magnitude of rotation, whereas DA levels in the NAcc impact upon the rate of locomotor activity, as the NAcc mainly drives behavioural activation, as discussed in Ref. [42]. Rats that received unilateral four-site intrastriatal 6-OHDA lesions, in which the NAcc and VTA were largely spared, rotated more at 6 weeks post lesion than MFB lesion rats [41]. Taken together, the involvement of the mesencephalic sub-regions in the rotational response to amphetamine can be summarised such that striatal dopamine levels mediate the direction of the response and those with spared mesocorticolimbic dopamine rotate higher. Therefore the VTA mainly drives locomotor activity. In the present study we have encountered the same case as in Ref. [52] in that striatal dopamine was not equally depleted across the groups and therefore the bundle lesion group produced the highest number of rotations. Once the influence of the SNc/VTA cell counts were accounted for in each of the respective correlations, it became clear that the corridor test and the amphetamine-induced rotation were exclusively correlated with SNc cell count and that the cylinder test was influenced more by the SNc than by the VTA. These results show that the influence of the VTA on the majority of tests is small and that behavioural performance is largely driven by SNc cell counts. On the corridor test and the amphetamine induced rotation, performance is driven exclusively via SNc cell counts whereas no single test exclusively correlated with VTA cell loss. Interestingly, spontaneous rotation and performance on the balance beam apparatus correlate more with cell counts in the VTA than cell counts in the SNc, revealing that these two tests are influenced by cell loss in both areas.

These results demonstrate (i) the sensitivity of the behavioural tasks to motor deficits of varying magnitude and (ii) that some of the behavioural impairments are differentially correlated with cell loss in the SNc and VTA. It has recently been suggested that the use of multiple tasks to assess the size of a unilateral 6-OHDA lesion is appropriate [13], specifically the use of drug-induced rotation, followed by simple behavioural tests such as the cylinder and/or the corridor test. Here we confirm the usage of the corridor test, the cylinder test and amphetamine-induced rotation in that they correlate highly with SNc cell count. Furthermore, we would extend this suggestion to include the balance beam test and spontaneous rotation and, in addition, extend the behavioural findings to the other two mouse lesion models of nigrostriatal dysfunction.

The present results are in accordance with previous work that characterised the role of the VTA and SNc and their primary target sites the NAcc and the striatum. In a comprehensive study Kirik et al. compared complete MFB lesions to striatal lesions of several different sites (1, 2, 3, and 4 site) on different behavioural tests in rats. Only rats with extensive DA depletion (MFB and 4-site striatal lesions) showed deficits on the staircase test and the stepping test. Cell loss in the VTA was less than 30% in all striatal lesion groups compared to 80% depletion in the bundle lesion group. The authors suggest that a behavioural impairment is seen only in severely depleted rats. In rats, a critical depletion level for behavioural impairments has been shown to occur at a lower level for amphetamine induced rotations (30–50% SNc cell loss)

compared to staircase test (60–80% SNc cell loss) [41]. Smaller depletions may lead to spontaneous behavioural recovery [52]. The behavioural impairment is largely dependent on three factors, the site of injection, the degree of depletion and the behavioural test used [41]. Whereas some tests are more sensitive to dorsolateral striatal dopamine depletion, as e.g. the drug induced rotation tests, others are more sensitive to ventromedial striatal dopamine depletion as locomotor activity.

### 4.4. Selection of mice based on behavioural performance

The results discussed above indicate the level of variability that our group experiences with each of the three lesion types respectively. We have described the histopathological and behavioural changes in these groups compared to untreated control mice. After selecting a subgroup of mice post hoc based on <20% remaining TH-ir cells in the SNc on the lesioned side of the brain we can see that these mice are impaired on a number of tests described above (see Table 6). These mice displayed a strong bias on the corridor test (23.1–16.4%) and greater difficulties to stay on the rotarod apparatus (< 77 s). Furthermore, lesioned mice rotated to challenges with amphetamine (78 rotations) and apomorphine (−32 rotations). The balance beam apparatus was especially useful in MFB (80% bias) and SN (89% bias) lesioned mice, whereas the cylinder test was more useful in the striatum (73% bias) and MFB (77% bias) lesion groups. The stability of the lesion has been shown by [13] for SN lesioned mice, when depletion exceeded 60% denervation of the striatum. Furthermore, the behavioural impairment described above is comparable to the criteria proposed by [13] for a selection of well-lesioned mice.

## 5. Conclusion

Despite the ease and frequency of use of the unilateral 6-OHDA lesion rat, there are significant complications involved in translating this model to the mouse. Mice are certainly more vulnerable following surgery but an intensive post-operative welfare regime keeps survival rates high. Lesion success is dependent on the site of injection: on the one hand, smaller targets (SNc, MFB) typically produce more variable lesion results, but on the other hand when well targeted they can result in more complete lesions. Here, we present several drug-induced and voluntary simple motor behavioural tasks that are sensitive to detect damage to the dopaminergic system in mice and on several tests performance correlates well with the extent of SNc cell loss, whereas only one test, cage lid turning, was preferentially associated with unilateral VTA cell loss. The present results allow for the selection of the most suitable lesion model and behavioural tests for pharmacological and cell replacement therapies in unilateral mouse models of PD.

## Conflict of interest

SBD receives a royalty from Campden Instruments on sales of the staircase paw reaching apparatus. The authors declare no other conflicts of interest.

## Acknowledgements

The study was funded by the EU Framework 7 NeuroStemCell and REPLACES collaborative programmes, the UK Biotechnology & Biological Sciences and Medical Research Councils, and Parkinson's UK.

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# Chapter 3.5

## Experiment 5: Comparison of 6-hydroxydopamine lesions of the substantia nigra and the medial forebrain bundle on a lateralised choice reaction time task in mice.

After the assessment of the initial work of characterising the motor impairments of unilateral infusion of 6-OHDA along the nigro-striatal pathway in mice we aimed to (i) further characterise the lesion model on a more complex behavioural task, i.e. the lateral choice reaction time task in the 9-hole box in mice, and (ii) investigate the stability of the lesion over time to assess the usefulness of the model for future transplantation studies.

Unpublished work by the author has shown that in a direct comparison of quinolinic acid lesioned mice, administered intrastriatally, and 6-OHDA lesioned mice, administered intra-nigrally, that using the lateralised choice reaction time task in mice is feasible for the analysis of lesion induced deficits. In this first study we assessed the task parameters (both hold and stimulus duration) and characterised the response profile of the lesion. Unfortunately the 6-OHDA SN lesions, which are relevant for the present thesis, did undergo spontaneous recovery on response accuracy within 3 weeks of post-lesion testing, whereas lesioned animals still displayed impairments in movement time latencies. The 6-OHDA SN lesion model was primarily chosen for this study because of the associated low mortality rates and the larger ‘completeness’ of the lesion compared to striatal lesions. However, because of the recovery seen, which was largely due to remaining DA innervation, this model was deemed not suitable for long-term behavioural studies. In the light of similar experiments in rats we opted for a direct comparison between SN lesioned mice with MFB lesioned mice on the choice reaction time task.

The experiment conducted in the present paper as well as analysis of the data, histology and preparation of the manuscript was undertaken entirely by myself. Professor S.B. Dunnett was involved in planning of the experiment and gave help and advice throughout as well as in the writing of the manuscript. Fellow PhD student Gaynor Ann Smith also assisted during surgery of the large number of animals involved.

EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

*European Journal of Neuroscience, Vol. 37, pp. 294–302, 2013* doi:10.1111/ejn.12036

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# Comparison of 6-hydroxydopamine lesions of the substantia nigra and the medial forebrain bundle on a lateralised choice reaction time task in mice

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*Keywords*: behaviour, dopamine, movement time, operant, Parkinson's disease, reaction time

## Abstract

Parkinson's disease is most commonly modelled via unilateral infusion of the neurotoxin 6-hydroxydopamine (6-OHDA) in the rat, but recent work has been aimed to translate the reproducibility and reliability of the model to the mouse. Here we present the effects of unilateral 6-OHDA lesions to either the medial forebrain bundle or the substantia nigra (SN) in mice, which were trained on a lateralised choice reaction time (RT) task. This task measures response accuracy as well as RT and movement time latencies, and offers the opportunity for a more fine-grained analysis of the precise nature of the movement deficit, motor learning and functional recovery than can be achieved using classical tests of simple motor asymmetry. Both lesion types caused impaired response accuracy, which was more pronounced when responses had to be directed contralateral to the lesion. Furthermore, movement times were increased for both lesion groups, whereas only the bundle lesion group displayed a RT deficit. The lesions were stable over three consecutive weeks of testing, therefore lesion-type and behavioural assessment on the operant task are suitable to investigate the dopaminergic system in parkinsonian mice. Both lesions were stable over time, and were more pronounced when responses were directed in contralateral space; the mice with more complete bundle lesions displayed a greater deficit than mice that received lesions to the SN. The translation of this choice RT task will be beneficial for the assessment of therapeutics in mouse models of the disease.

## Introduction

Parkinson's disease (PD) is most commonly modelled in the rat via unilateral injection of the neurotoxin 6-hydroxydopamine (6-OHDA) at various levels of the medial forebrain bundle (MFB), which leads to subsequent degeneration of catecholaminergic cells in the substantia nigra (SN; Ungerstedt, 1968; Ungerstedt & Arbuthnott, 1970; Kirik *et al.*, 1998; Deumens *et al.*, 2002; Bove *et al.*, 2005) and a subsequent loss of dopamine (DA) in the striatum (Blandini *et al.*, 2000, 2007; Grealish *et al.*, 2008), which in turn gives rise to the cardinal symptoms of PD (Evarts *et al.*, 1981; Berardelli *et al.*, 2001; Shahed & Jankovic, 2007). Although the unilateral 6-OHDA rat lesion model has been used extensively to model PD and potential treatments such as pharmacological and cell replacement therapies, relatively little research has been conducted in mice.

As model organisms, mice have the advantage that genetic modifications are more readily achievable, allowing manipulation of genes that have been linked to sporadic PD in man (Alvarez-Fischer *et al.*, 2008; Duty & Jenner, 2011; Blandini & Armentero, 2012). To circumvent the high mortality rates in mice with MFB lesions (Lundblad *et al.*, 2004; Grealish *et al.*, 2010), striatal and nigral lesions have been utilised (Lundblad *et al.*, 2004, 2005; Cenci & Lundblad, 2007; Grealish *et al.*, 2010), resulting in better survival, but less complete lesions both of the neostriatum itself and with sparing of fibres that originate in the adjacent ventral tegmental area (VTA; Carli *et al.*, 1989; Kirik *et al.*, 1998; Grealish *et al.*, 2008). In a recent study, the three main lesion sites (SN, MFB, intra-striatal) have been compared on a series of simple motor behavioural tests in mice, and increased survival rates were achieved by intensive post-operative caring regimes (Francardo *et al.*, 2011; Heuer *et al.*, 2012; Smith *et al.*, 2012).

Unilateral 6-OHDA lesions have been shown to produce an asymmetry on behavioural tests measuring motor output, such as turning-behaviour, paw preference and increased movement times (Ungerstedt & Arbuthnott, 1970; Schallert *et al.*, 1978; Carli *et al.*, 1985, 1989; Torres & Dunnett, 2007; Grealish *et al.*, 2010; Heuer *et al.*, 2012; Smith *et al.*, 2012). Although tests of simple motor behaviour are relatively inexpensive in terms of costs and time (Francardo *et al.*, 2011; Heuer *et al.*, 2012; Smith *et al.*, 2012), more complex functions that can allow more precise dissection of the precise nature of the motor impairment are rarely assessed. Operant analysis of animals' behaviour provides a high level of stimulus control, objective gathering of data, high throughput testing and the ability to collect systematically a broader range of data parameters. Furthermore, this present task is dependent on goal-directed behaviour, and allows for the assessment of higher cognitive functions (including response selection and

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*Received 20 July 2012, revised 21 September 2012, accepted 23 September 2012*

response initiation), as well as of motivation, working memory and attention. In the context of the 6-OHDA model of PD, such tasks have been used to investigate the functional nature of a deficit, for example whether the impairment is due to a sensory-input or a motor-output deficit (Carli *et al.*, 1985; Dowd & Dunnett, 2005a,b). In the rat, the lateralised choice reaction time (RT) task introduced by Carli *et al.* (1985) proved sufficiently sensitive to detect small changes in behaviour where the simple motor assessments failed to measure improvements (Carli *et al.*, 1985, 1989; Dowd & Dunnett, 2004). Unilateral lesioned rats display impairments in response accuracy, increased RTs and increased movement times when the required response had to be directed towards the side contralateral to the lesion (Carli *et al.*, 1985; Brasted *et al.*, 1998; Dowd & Dunnett, 2005a,b).

Assessment of sensory-motor outputs by the lateralised choice RT task has not hitherto been achieved in mice. Here we compare two of the unilateral mouse lesion models (SN vs. MFB) on this operant task to assess the lesion-induced deficit as well as the stability of the lesion over time. The stability of a repairable deficit is fundamental to the development and assessment of therapies without the danger of spontaneous recovery interfering with the results.

## Materials and methods

### Subjects

Fifty-eight male mice of the C57/Bl6 strain were used. All mice were housed in standard laboratory cages in pairs of one-two in a temperature- and humidity-regulated room (21 ± 2 °C) on a 10 : 14 h dark–light cycle, with lights on at 06:00 h. All animals had access to food and water *ad libitum*, except during the testing phases that required food restriction for motivation. All testing was done in accordance with local ethical review and approval from Cardiff University and licences according to the United Kingdom Animals (Scientific Procedures) Act, 1986.

### Experimental design

One week after arrival all mice were food-restricted for 1 week before starting training on the operant task (see below). After the mice had learned the choice RT task to an asymptotic level of performance, the animals were matched on their accuracy into three groups. Two of the groups underwent lesion surgery (SN and MFB), whereas one group served as unlesioned control. Four weeks after the lesion the rotational response to 2.5 mg/kg methamphetamine was recorded and then the animals were again food-restricted. After 3 weeks of operant testing (weeks 5–8 post-lesion), all mice were perfused for histological analysis.

### Drug-induced rotation

Rotational behaviour was assessed in automated rotometer bowls, designed after the model by Ungerstedt (Ungerstedt & Arbuthnott, 1970). Mice were injected with methamphetamine (2.5 mg/kg; Sigma) and placed in the rotometer bowl for 60 min. Data were expressed as average net number of rotations per min.

### Operant tests

#### Apparatus

Testing was conducted in a bank of 16 identical nine-hole boxes (CeNeS, Cambridge, UK). In brief, each operant chamber was fitted with a curved array of nine response holes on one side of the chamber and a magazine on the opposite side. Each of the response holes was fitted with infrared-sensors that could detect entries into the respective hole. Through a peristaltic pump 5 μL of Strawberry milk (Yazoo®, Campina, UK) was delivered into the magazine as a reward. All boxes were placed in sound-attenuating cubicles and controlled by a Camden controlled software system (BNC Control, Campden Instruments, Loughborough, UK).

### Choice RT task

For testing, all animals were food-restricted to 90% of their free feeding weight 1 week before the start of operant testing by giving the animals weighed amounts of food into their home-cages approximately 1 h after the respective testing session. All mice were weighed regularly and the daily allocation of food was adjusted to maintain body weight at approximately 90% of their free feeding weight.

The choice RT task used in the present experiment has been translated from a rat version that has been described elsewhere (Carli *et al.*, 1985; Dowd *et al.*, 2005). In the response array only three holes were available for the mice to respond, which were holes 3, 5 and 7, whilst all the other holes were covered. In brief, each trial started with illumination of the centre hole light (hole 5) whilst all other lights were switched off. If the infrared beam in front of the response hole was broken by the entry of the animals' nose into the hole a timer was started. Randomly chosen by the computer, the mouse had to sustain its nose-poke by one of the following four delays: 25, 50, 75, and 100 ms. Premature withdrawal from the response hole resulted in abortion of the trial and 'punishment' of 5 s in darkness as timeout interval. Each procedural error resulted in the random selection of a new trial. If the hold was sustained for the required duration, one of the two lateralised response locations (randomly chosen) located to either side of the animals head (holes 3 and 7) was illuminated briefly (300 ms). The animal then had 5 s to make a response. A poke into the previously illuminated response hole would result in the delivery of a reward into the illuminated magazine, whereas an incorrect response or no response resulted in abortion of the trial and start of the 5-s timeout period. After the timeout period or after the mouse had collected its reward from the magazine, the houselight was switched on (and magazine light switched off in the case of previous reward) for 2 s as inter-trial interval. After the inter-trial interval finished a new trial was started. Each daily session was 30 min long.

The main parameters that were recorded were the total number of trials started (TTS); the total number of trials usable (TTU; i.e. the number of trials in which the animals sustained their nose-poke for the required delay period); response accuracy (Accuracy), as the percentage of correct responses divided by the number of usable trials, expressed as a percentage; RT, defined as the time recorded from onset of the lateralised stimulus presentation until retraction of the animals' nose from the centre hole; and movement time, which was defined as the latency from retraction of the centre hole until the execution of the lateralised response.

### Pre-training operant task

All mice were trained in stages to the final choice RT task outlined above. All animals were food-restricted 1 week before operant training started. On the first day of training, the mice were placed into the operant boxes for 30 min with the houselights switched on and 1 mL of reward placed in the food magazine to habituate the animals to the chamber and to teach them the location of the reward delivery. On the second stage of training, all lights with the exception of the

magazine light were switched off and 5 μL of reward was delivered into the magazine. If the animal collected the reward, the magazine light was extinguished and the houselight was switched on for the duration of 10 s, upon which a new trial started. All mice learned to collect the reward from the magazine within one session. On the third stage of training all lights were switched off, with the exception of the stimulus light in response hole 5. A fixed ratio schedule of reinforcement (FR1) was introduced so that every poke into the illuminated response hole would result in the delivery of 5 μL reward into the illuminated magazine. For some mice the response hole was baited with a drop of strawberry milk to facilitate learning, but all mice displayed a high number of responses (137 ± 10) to the response hole on day 6 of training. During the next stage of training, a poke into hole 5 resulted in the illumination of response hole 3 or 7 (randomly determined by the computer). A response into the second illuminated hole would then result in reward delivery. Over 4 weeks of training the duration of the lateralised stimulus was gradually reduced from 5000 to 300 ms, and the duration the mouse had to hold its nose in the centre hole before illumination of the lateralised response holes was increased from 5 to 200 ms. For the longest delay of 200 ms only 20% of all trials were usable and therefore the following shorter delays were chose for the final task outlined below: 25, 50, 75 and 100 ms.

### *Surgery*

Mice were anaesthetised in an induction chamber under 1.5–2% isoflurane using $O_2$ as carrier gas. Following shaving and cleaning of the surgical area, the animal was placed in a stereotaxic frame (Kopf) and anaesthesia was maintained under 1.5–2% isoflurane in a 2 : 1 mixture of $O_2$/NO. Bregma was exposed via incision of the skin, and a small burr hole was created using a dental drill to which the lesion cannula was lowered to either of the following stereotaxic coordinates: SN (1.5 μL): AP −3.0, ML −1.2 and DV −4.5; or MFB (1.0 μL): AP −1.2, ML −1.2 and DV −4.75. The nose bar was set at 0.0 mm. 6-OHDA (Sigma) was infused via a 30-gauge steel cannula that was connected via polyethylene tubing to a 10-μL Hamilton syringe mounted on a micro-drive pump. The toxin was used at a concentration of 6 μg/μL (hydrobromide salt, calculated from free-base weight) dissolved in 0.9% sterile saline with 0.2 mg/mL ascorbic acid. Infusions were done at a rate of 0.5 μL/min, and the needle was left in place for an additional 2 min to allow for diffusion before retraction of the needle and suturing the wound. During surgery the mice would receive s.c. injections of 0.03 mL Metacam® (Meloxicam, Boehringer, Ingelheim, Germany) as analgesia and 1 mL of 4% glucose–saline solution.

The animals' body weight was monitored for 14 days post-lesion, and all lesioned animals received injections of 0.5 mL glucose–saline s.c. three times daily, and wet palatable food was provided in small containers on a daily basis.

### *Histology*

After completion of behavioural testing all animals were deeply anaesthetised with 0.2 mL sodium pentobarbitone, and perfused through the heart with approximately 25 mL phosphate-buffered saline (PBS; pH 7.3) followed by 50 mL 1.5% paraformaldehyde in PBS. The brains were then post-fixed for a further 24 h before being transferred into a 25% sucrose solution where they were kept until sunk. Thereafter all brains were cut into coronal sections (40 μm) on a freezing sledge microtome into Tris-buffered saline (TBS; pH 7.3) with 2% azide and stored until further processing.

A 1 : 6 series of sections was washed in TBS before quenching endogenous peroxidase activity in a 3% $H_2O_2$ and 10% methanol solution in distilled water for 10 min. After 3× washing and re-equilibrating in TBS, sections were incubated for 1 h in 3% normal horse serum block in TXTBS (TBS with 0.2% Triton-X) before transferring them into primary antibody solution [tyrosine hydroxylase (TH), raised in rabbit; Chemicon, 1 : 1000] and left at room temperature overnight. After washing the sections they were incubated for 3 h in biotinylated secondary antibody in TBS and 1% serum. Following washing in TBS, the sections were transferred into a Dako streptavidin ABC kit (Vectastain; Vector Laboratories, Peterborough, UK) in 1% serum for 2 h. After washing 3× in TBS and twice in TNS (Tris non-saline; pH 7.4), the antibodies were visualised by staining with diaminobenzidine (Sigma) at a concentration of 0.5 mg/mL in TNS with 3% $H_2O_2$. Stained sections were washed in TNS and TBS and mounted on gelatine-coated glass slides. After mounting, all slides were air-dried overnight and dehydrated in an ascending series of alcohols (70, 95, 99%; 5 min each) and xylene before coverslipping using a DPX mounting media.

### *Optical density (o.d.)*

o.d. of dopaminergic fibres was assessed as described previously (Grealish *et al.*, 2010; Heuer *et al.*, 2012). In brief, coronal pictures of TH-immunoreactive (ir) sections were taken across four levels through the striatum (approximately from bregma: +1.10, +0.62, +0.14 and −0.34 mm) using a Leica DM/RBM microscope under 1× magnification. Using IMAGEJ software (Version 1.42; NIH, USA) o.d. was measured in all sections, correcting for non-specific staining by subtracting values from measures at the corpus callosum. o.d. was expressed as percentage of the side contralateral to the lesion. Anatomical landmarks to divide the striatum into dorsal and ventral parts were made by drawing a horizontal line across the most dorsal point of the lateral ventricle or the anterior commissure, where visible (Grealish *et al.*, 2010).

### *TH-ir cell counts*

TH-ir cells were counted at ×10 magnification under a Leica DM/RBE microscope. Cell counts were made for ipsilateral and contralateral SN and VTA in one section. The medial terminal nucleus of the accessory nucleus of the optic tract was used to select the section and to define the boundary between VTA and SN cells (Dowd & Dunnett, 2005a). All cell counts are expressed as percentage of the contralateral side.

### *Statistics*

Behavioural and histological data were analysed using the GENSTAT (Version 12.1) statistical software package. Operant data were analysed using repeated-measures analysis of variance (ANOVA), and all other data were analysed using one-way ANOVA or Pearson correlations. *Post hoc* comparisons between groups were done using Student's–Newman–Keuls test where appropriate. A significance level of $\alpha = 0.05$ was used to determine if *F*-ratios were significantly different from normal.

## Results

### *Mortality and group sizes*

Of the 58 mice that were used in the present experiment, six animals died during the course of testing. Four mice died shortly after the lesion surgery (two from each group), whereas the remaining

two deaths appeared to be unrelated to the procedure. Of the 46 mice that received lesions, 32 mice were allocated to the MFB lesion group and 14 mice were allocated to the SN lesion group. Survival rates were at acceptable levels and comparable to previous reports from our group (Heuer *et al.*, 2012; Smith *et al.*, 2012).

After o.d. analysis and inspection of the TH-ir stained sections, we excluded animals that were regarded as partially lesioned on the basis of an o.d. score of > 35% and a degeneration of TH-ir cells in the ipsilateral SN at maximum 20% remaining cells. The final group sizes used for the analysis were: Control, $n = 12$; MFB, $n = 19$; and SN, $n = 8$ mice per group.

### 6-OHDA lesions cause degeneration of TH-ir cells

Injection of 6-OHDA into both, the SN or the MFB resulted in a degeneration of TH-ir cells in the SN. As shown in Fig. 1, both types of lesions reduced TH-ir staining in the dorsal neostriatum and in the ipsilateral SN. Although ventral striatal areas and the VTA showed marked loss of TH-ir staining, the MFB lesions produced the larger overall depletion of TH-ir cells.

The lesion induced a reduction in TH-ir density on the side of the lesion (Fig. 2A; Group, $F_{2,36} = 113.97$, $P < 0.001$). *Post hoc* comparisons showed that both lesion groups had density scores that were significantly lower than those of the controls ($P < 0.001$). Although overall o.d. was 10% higher in the SN lesion group compared with the MFB lesion group, this difference did not reach statistical significance (n.s.). Dorsal striatal o.d. was significantly different between the three groups (Fig. 2B; Group, $F_{2,36} = 132.05$, $P < 0.001$), with both lesion groups displaying reduced TH-ir density compared with those of the control group (both $P < 0.001$). Ventral striatal density was significantly different between the three groups (Fig. 2C; Group, $F_{2,36} = 84.25$, $P < 0.001$), with both lesion groups showing reduced o.d. compared with the control group (both, $P < 0.001$). The MFB lesion group displayed a higher reduction in ventral striatal TH density compared with the SN lesion group (MFB = 21.1%; SN = 39.6%, $P < 0.05$).

There was no difference in the total number of TH-ir cells in the contralateral SN (Group, $F_{2,36} = 0.45$, $P$ = n.s.) and in the contralateral VTA (Group, $F_{2,36} = 0.16$, $P$ = n.s.) between the three groups, therefore cell counts were expressed as a percentage of the contralateral side for all groups. Counting TH-ir cells in the midbrain revealed a significant loss of cells induced by the 6-OHDA infusion (Fig. 2D; Group, $F_{2,36} = 105.21$, $P < 0.001$), with both lesion groups displaying a significant reduced number of TH-ir cells compared with the control group (both, $P < 0.001$). Both types of lesions were effective in producing a near-complete depletion (< 10% cells remaining) of TH-ir cells in the ipsilateral SN (Fig. 2E; Group, $F_{2,36} = 241.35$, $P < 0.001$), with both lesion groups differing significantly from control (both, $P < 0.001$). TH-ir cells in the VTA were less affected than the cells in the SN, but compared with control there was a marked reduction in cell numbers in both lesion groups (Fig. 2F; Group, $F_{2,36} = 38.95$, $P < 0.001$). However, animals of the MFB lesion group displayed a higher loss of cells in the VTA compared with animals of the SN lesion group (10.5% more cells lost), but this difference failed to reach statistical significance.

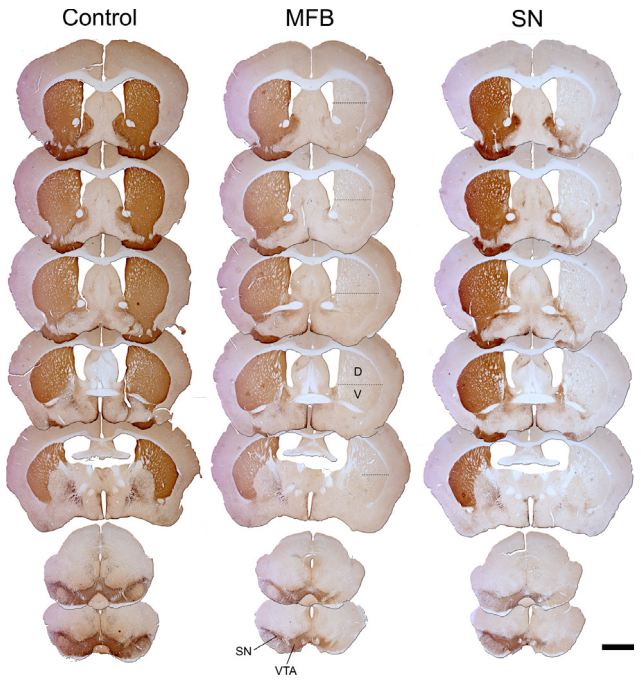


FIG. 1. Representative series of TH-ir stained sections for control, medial forebrain bundle (MFB) and substantia nigra (SN) lesion groups, respectively. The dotted line indicates the separation of the striatum into dorsal (D) and ventral (V) subdivisions used for o.d. analysis. Scale bar: 1 mm.

### Behavioural changes

#### *Amphetamine-induced rotations*

There was a difference between the groups in the average number of net rotations in response to 2.5 mg/kg amphetamine (Fig. 3; Group, $F_{2,36} = 6.99$, $P < 0.01$). *Post hoc* analysis showed that both lesion groups rotated consistently higher to amphetamine than animals of the control group (both, $P < 0.01$), with no difference between the lesion groups.

### Choice RT task

#### *TTS and TTU*

During baseline assessment all animals started a high number of trials by responding to the illuminated centre hole with a nose-poke (Fig. 4A). The lesion caused a significant reduction in the TTS in both lesion groups (Fig. 4A; Week × Group, $F_{3,108} = 34.67$, $P < 0.001$), with the most pronounced reduction seen with MFB lesions. All *post hoc* comparisons demonstrated that during each of the 3 weeks of post-lesion testing both lesion groups attempt significantly fewer trials compared with the control group, and during all 3 weeks of post-lesion assessment the MFB-lesioned animals attempt fewer trials than animals of the SN lesion group (all $t > 4.37$, $P < 0.01$).

Once a trial is started the animal has to sustain the nose-poke for the required delay in order to be presented with the lateralised stimulus, to which it has to respond. Therefore there are three types of procedural errors the mouse can make in order to make a trial invalid: (i) premature withdrawals; (ii) repeated centre hole pokes; and (iii) not responding to the lateralised stimulus within the allocated period of time (5 s). The number of trials that are started (see above) is therefore rather a measure of motivation of the animal rather than performance on the operant task. During baseline assessment animals of all three groups produced a similar number of usable trials (Fig. 4B), approximately 63% of the trials started were usable during baseline testing. After the lesion, both lesion groups produced fewer usable trials than mice in the control group

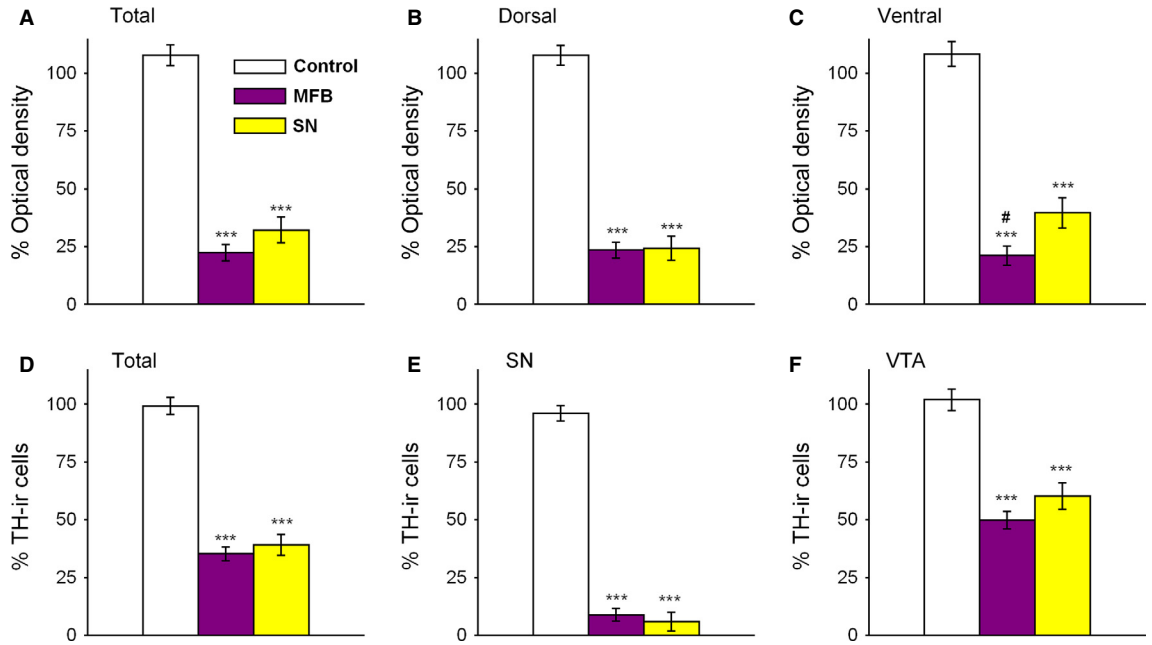


FIG. 2. Optical density (o.d.) and cell counts. Average striatal o.d. for (A) the whole, (B) the dorsal, and (C) the ventral neostriatum on the lesioned side, in each case expressed as a percentage of the contralateral side. Tyrosine hydroxylase-immunoreactive (TH-ir) cells were counted at one level in the midbrain and expressed as a percentage of contralateral cell counts for (D) the total cell loss, (E) cell loss in the substantia nigra (SN), and (F) cell loss in the ventral tegmental area (VTA). The number of asterisks above the bar charts denote significant differences at the < 0.05, < 0.01 and < 0.001 levels of significance, respectively. MFB, medial forebrain bundle.

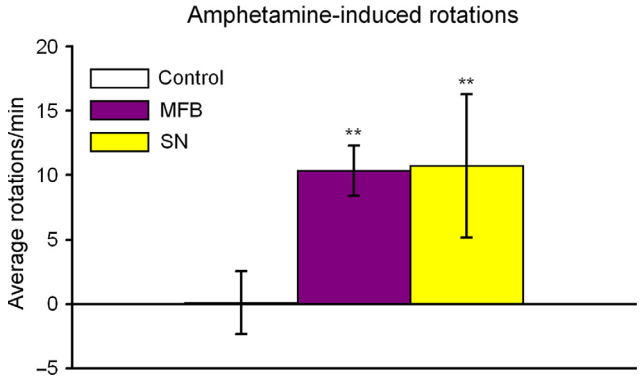


FIG. 3. Performance on the amphetamine-induced rotation test in response to 2.5 mg/kg amphetamine per group. Asterisks denote significantly different from control at the < 0.01 level of significance. MFB, medial forebrain bundle; SN, substantia nigra.

(Week × Group, $F_{3,108} = 32.52$, $P < 0.001$). The number of TTU was similar to the TTS, and significantly different between the two lesion groups and the control group, as well as between the two lesion groups, with the MFB group producing the smallest number of usable trials (all, $t > 6.29$, $P < 0.01$). Most of the usable trials were produced when the delay between the initial centre hole poke and the onset of the lateralised stimulus was short. All rats had a higher efficiency [Efficiency % = (TTU/TTS)*100] of usable trials when delays were short (Delays, $F_{3,108} = 278.18$, $P < 0.001$). This effect was more pronounced in animals of the lesion groups, which produce fewer usable trials as a proportion of trials started (Group × Delay, $F_{6,108} = 14.32$, $P < 0.001$), and effect that was only different from control after the lesion and not during baseline testing (Weeks × Group × Delay, $F_{18,324} = 8.44$, $P < 0.001$).

*Accuracy*

Response accuracy was defined as the number of correct trials divided by the number of usable trials and expressed as a percentage. During baseline assessment all animals responded with a high overall (i.e. ipsilateral and contralateral combined) accuracy towards the lateralised stimulus (90% correct). After the lesion, animals of both lesion groups showed impaired response accuracy, whereas the control group continued to respond with > 90% accuracy during all 3 weeks of post-lesion assessment (Fig. 4C; Week × Group, $F_{6,108} = 21.61$, $P < 0.001$). Both lesion groups performed with lower response accuracy than the control group during all three time-points of post-lesion assessment (all, $t > 4.74$, $P < 0.01$), with the exception of the SN lesion group in the third week of post-lesion assessment. Although the SN lesion group performed with lower accuracy compared with the control group ($t = 3.89$, $P < 0.05$), accuracy increased in this group from 63% correct trials during week 1 post-lesion to 77% correct trials during week 3 post-lesion. During all 3 weeks of post-lesion assessment the MFB lesion group performed worse than animals in the SN lesion group (all, $t > 6.82$, $P < 0.01$). Although response accuracy was lower overall in lesioned animals (i.e. responses to both sides were impaired), the impairment was more pronounced when the required response had to be directed towards the hole located on the contralateral side of the lesion (Fig. 5A; Week × Side × Group, $F_{6,108} = 6.32$, $P < 0.001$). Although the most impaired lesion group, the MFB lesion group, was still able to respond to ipsilateral presented stimuli with 63% average response accuracy over the 3 weeks of post-lesion assessment, accuracy dropped to 16–25% when responses had to be directed into contralateral space. The average difference between ipsilateral and contralateral response accuracy was 43.5% in the MFB lesion group. Responses for the SN lesion group were lateralised as well, with responses to the ipsilateral side being not

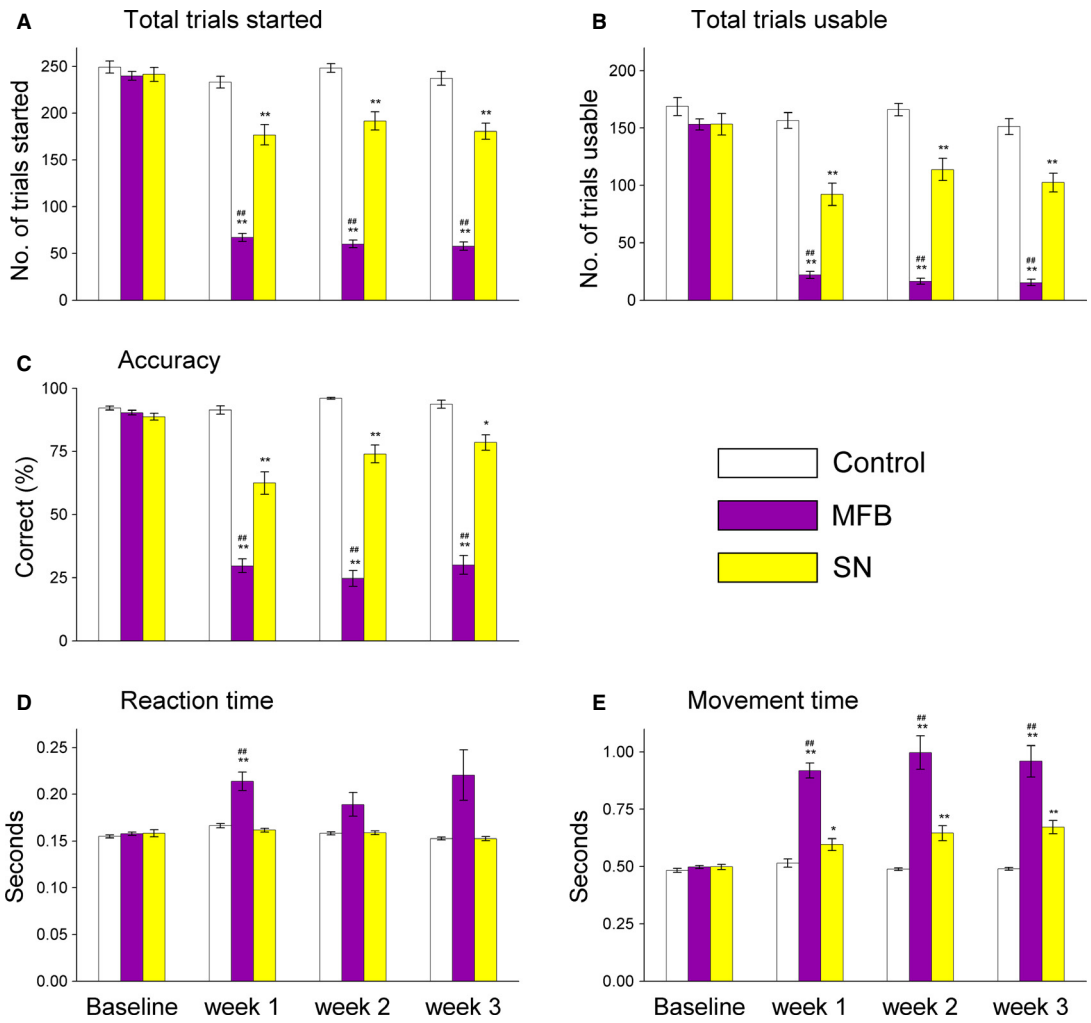


FIG. 4. Main effects of the choice RT task by week of testing. The data are collated over 5 consecutive days as baseline and three blocks of 5 consecutive days post-lesion (weeks 1–3). (A) TTS. (B) TTU, i.e. trials in which the animals sustained the nose-poke for the required delay and executed the lateralised response. (C) Response accuracy (% correct) on usable trials. (D) RT on correct trials. (E) Movement time on correct trials. The number of symbols denotes significant differences at the < 0.05 and < 0.01 levels of significance. Asterisks denote significantly different from control, whereas hashes denote significantly different between the two lesion groups, as shown by Student's–Newman–Keuls *post hoc* analysis. MFB, medial forebrain bundle; SN, substantia nigra.

different from control (all, $t < 1.27$, $P$ = n.s.). As can be seen in Figs 4C and 5A, the average difference in accuracy between the two sides of responding was 28.9% for the SN lesion group, and therefore 14.6% higher than the MFB lesion group difference.

### *RTs and movement times*

The time to withdraw the nose upon detection of the lateralised stimulus was defined as the RT, whereas the time taken to execute the lateralised response was defined as the movement time. Whereas there was no difference between the three groups during baseline assessment in either of the two response latency measures (Fig. 4D), after the lesion RTs were increased in animals that received lesions to the MFB only (Group, $F_{2,36} = 10.90$, $P < 0.001$). The increase in RT was not lateralised (Fig. 5B; Week × Sides × Group, $F_{6,108} = 1.39$, $P$ = n.s.) as the MFB lesion group took longer to withdraw its nose in response to the lateralised stimulus irrespective of the side of stimulus presentation.

The time to execute the lateralised response, i.e. the movement time, was prolonged after the 6-OHDA lesion (Fig. 4E; Weeks × Group, $F_{6,108} = 13.48$, $P < 0.001$). Both lesion groups were taking longer to respond than control mice, but mice of the MFB lesion group were more impaired than those in the SN lesion group. Both lesion groups took longer for the execution of the lateralised response when responding to the side contralateral to the lesion (Fig. 5C; Weeks × Sides × Group, $F_{6,108} = 10.64$, $P < 0.001$). When responding to the ipsilateral side of the lesion, only the MFB group was slower in responding than the control group, whereas the SN group had similar response times to the controls.

### *The behavioural data correlate with the extent of TH-ir cell loss in the SN*

TH-ir cell counts in the SN correlated well with o.d. in the dorsal striatum (Fig. 6A; $r = 0.932$, $P < 0.01$), and cell counts in the VTA correlated highly with ventral striatal o.d. (Fig. 6B; $r = 0.730$, $P < 0.01$). The behavioural performance of the animals and their correlations with the extent of cell loss in the SN are shown in Fig. 6C–F; amphetamine-induced rotations correlated with percentage of cells remaining in the SN ($r = -0.512$, $P < 0.01$). High correlations were also found between remaining cells in the SN and response accuracy ($r = 0.671$, $P < 0.01$) and movement time

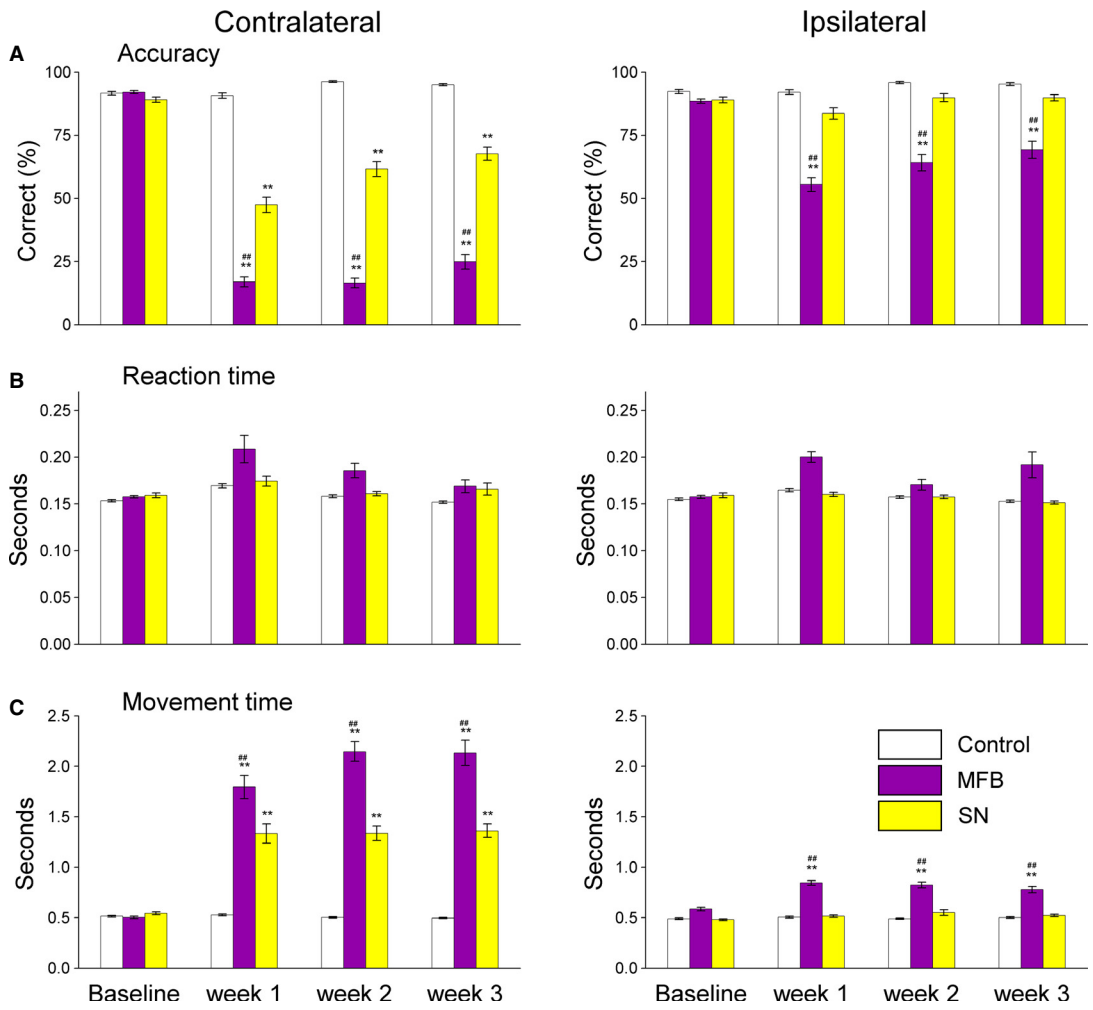


FIG. 5. Main effects of the operant choice RT task separated for ipsilateral and contralateral responses per block of testing. The data are collated over 5 consecutive days as baseline and three blocks of 5 consecutive days post-lesion (weeks 1–3). (A) TTS. (B) TTU, i.e. trials in which the animals sustained the nose-poke for the required delay and executed the lateralised response. (C) Response accuracy on correct trials. (D) RT on correct trials. (E) Movement time on correct trials. The number of symbols denotes significant differences at the < 0.05 and < 0.01 levels of significance. Asterisks denote significantly different from control, whereas hashes denote significantly different between the two lesion groups as shown by Student's–Newman–Keuls *post hoc* analysis. MFB, medial forebrain bundle; SN, substantia nigra.

($r = -0.512$, $P < 0.01$). RT only correlated weakly with the SN cell count ($r = -0.297$, $P < 0.05$).

## Discussion

The present study has shown the effects of two unilateral mouse lesion models of PD on a lateralised choice RT for the first time, and has defined the parameters to assess unilateral lesions to the nigro-striatal system in mice. The lesion caused a reduction in the number of TH-ir cells in the midbrain and subsequent loss of TH-ir staining in the striatum.

Mice with infusions of 6-OHDA into the MFB appeared to have a greater TH-ir depletion than SN-lesioned animals. This was confirmed by a statistically significant greater reduction of TH-ir o. d. in the ventral striatum of the MFB lesion group compared with the SN lesion group, although VTA cell counts were not statistically different between the groups. The lesion was effective in inducing impairments on multiple parameters of the operant task, with MFB-lesioned mice being more impaired than those that received SN lesions.

This study replicates the deficits that have been shown in unilateral 6-OHDA lesion rat models of PD (Dowd & Dunnett, 2005a,b), and demonstrates the validity of this task for mice. Near-complete unilateral dopaminergic lesions as achieved via infusion of 6-OHDA into the MFB led to an impairment in the TTS, TTU, a reduction in response accuracy, and increase in RT and movement time latencies (Carli *et al.*, 1985; Brown & Robbins, 1989; Dowd & Dunnett, 2004, 2005a,b). Lesions to the SN in the mouse produced a behavioural profile that was more similar to a striatal lesion in the rat (Carli *et al.*, 1985; Darbaky *et al.*, 2003; Dowd & Dunnett, 2005a,b). Although the SN-lesioned animals were impaired on all parameters, with the exception of RT latencies, the behavioural impairment was less pronounced compared with MFB-lesioned mice. In the rat the difference in response profile has been attributed to the terminal lesion being less complete and having more sparing of cells located in the VTA than those of the MFB lesions (Dowd & Dunnett, 2005a,b). Dowd & Dunnett proposed that the difference in behavioural profile lies in either the difference of lesion extent and/or in MFB lesions affecting additional structures compared with the terminal lesions (Carli *et al.*, 1985; Dowd & Dunnett, 2005a,b). Interestingly, combined lesions of

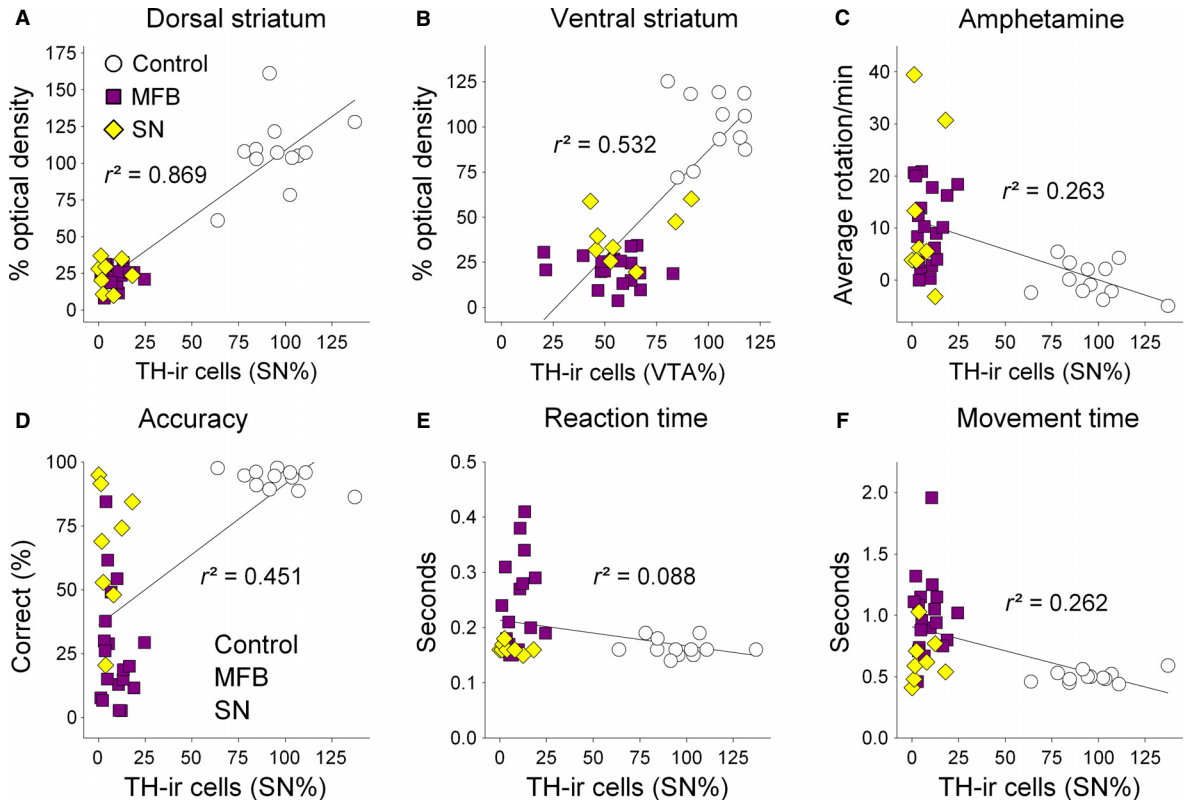


FIG. 6. Correlations between TH-ir cell counts and optical density measures in the dorsal (A) and ventral striatum (B) as well as correlations with behavioural outcome measures and SN TH-ir cell counts on Amphetamine-induced rotations (C), Accuracy (D), Reaction time (E) and Movement time (F).

the striatum and subsequently the nucleus accumbens do not have additive effects (Carli *et al.*, 1989). However, on a simple RT task conducted in the Skinner box, the larger the DA depletion in bilateral MFB-lesioned rats, the greater was the manifestation of RT and movement time impairments (Smith *et al.*, 2002).

In the initial reports by Carli *et al.* (1985), using rats with striatal 6-OHDA lesions demonstrated no impairment in movement time. In contrast, Dowd & Dunnett showed movement time deficits in both MFB- and striatal-lesioned rats, which is in line with the present findings (Dowd & Dunnett, 2005a,b). It has been argued the differences in movement time deficits are due to the configuration of the response holes, which were directly adjacent to the centre hole in the initial studies (Carli *et al.*, 1985), but were moved 'one-hole to the side' in the subsequent experiments (Dowd & Dunnett, 2004, 2005a; Dowd *et al.*, 2005). An alternative explanation was that the initial Carli studies had lesions that were less complete, as only one infusion site was used compared with four-site terminal injections (Carli *et al.*, 1985; Dowd & Dunnett, 2005a,b), which produce more widespread dopaminergic depletion (Kirik *et al.*, 1998).

As in previous reports, many of the outcome measures on the operant task were impaired for stimuli presented to either side of the body, but with the impairment being more pronounced when responses had to be directed into the hole contralateral to the lesion. Most importantly, the lesion-induced deficit was stable over time in both lesion groups. Whereas striatal lesions in the rat led to spontaneous recovery on some parameters of the choice RT task (Accuracy, Ipsilateral bias; Carli *et al.*, 1985; Dowd & Dunnett, 2005a), both lesions used in the present study were stable over three blocks of testing. There was some improvement in response accuracy during week 3 post-lesion in SN-lesioned mice, but the group was still performing significantly lower than mice of the control group. The severe motor and motivational impairment that was induced in MFB-lesioned mice can complicate interpretation of the deficit in accuracy. Nevertheless, even though the overall response rate was greatly reduced in MFB-lesioned mice, the pattern of responding was highly stable over the days of testing.

Interestingly, mice with lesions to the SN did not show the typical increase in choice RT that has been described in rats after unilateral DA depletion in the striatum after infusions of 6-OHDA (Carli *et al.*, 1985, 1989; Dowd & Dunnett, 2005a,b) or the MFB (Dowd & Dunnett, 2004, 2005a,b). By contrast, the only study utilizing SN lesions on a similar choice RT task did not report any differences in RT or movement time latencies (Darbaky *et al.*, 2003). In humans the RT deficit of patients with PD is also less clear, with some studies reporting increased RTs whereas others do not (Evarts *et al.*, 1981; Bloxham *et al.*, 1984; Rafal *et al.*, 1984; Brown & Marsden, 1986; Girotti *et al.*, 1986; Jordan *et al.*, 1992; Kutukcu *et al.*, 1999). Furthermore, the emergence of deficits seems to be largely dependent on the task and apparatus used (Jordan *et al.*, 1992; Gauntlett-Gilbert & Brown, 1998).

In conclusion, the current protocol of the lateralised choice RT task in mice is a useful method to assess lesion-induced deficit in unilateral mouse models of PD. Furthermore, future studies can assess the effects of therapeutic interventions, as cell replacement therapies, pharmacological or surgical approaches, or protection strategies (Dowd & Dunnett, 2004; Dowd *et al.*, 2005), as has been done in similar rat models, with the additional benefit to use mouse-derived stem cells without the need for immunosuppression or to lesion transgenic mice. This is the first demonstration of choice RT performance in mice showing that under the presented task parameters operant assessment in mice is a powerful tool for behavioural analysis. Furthermore, although simple motor tests have been defined recently

by others (Lundblad *et al.*, 2004; Iancu *et al.*, 2005; Grealish *et al.*, 2010) and our own group (Heuer *et al.*, 2012; Smith *et al.*, 2012), this is the first demonstration using the MFB and the SN unilateral mouse 6-OHDA lesion model on a more complex behavioural task. The stability of the lesion over the period of testing will provide useful for the assessment of cell-based therapies in the near future.

## Acknowledgements

This study was funded by the EU Framework 7 NeuroStemCell collaborative programme (EU Seventh Framework under grant agreement nr. 222943). The authors declare no conflict of interests. The authors thank Hanna Lindgren for comments on the manuscript.

## Abbreviations

6-OHDA, 6-hydroxydopamine; DA, dopamine; ir immunoreactive; MFB, medial forebrain bundle; o.d. optical density; PBS, phosphate-buffered saline; PD, Parkinson's disease; RT, reaction time; SN, substantia nigra; TBS, Tris-buffered saline; TH, tyrosine hydroxylase; TNS, Tris non-saline; TTS, total number of trials started; TTU, total number of trials usable; VTA, ventral tegmental area.

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# Chapter 3.6

## Experiment 6: Behavioural recovery on simple and complex tasks by means of cell replacement therapy in unilateral 6-OHDA lesioned mice

After the assessment of the simple and more complex behavioural analysis of the lesion induced deficits the aim of the present experiment was to investigate the restorative abilities of cell replacement therapies on the tests that have previously shown to be sensitive to DA depletion. Previous work conducted in rats reported that DA grafts derived from primary fetal tissue were able to restore some of the lesion induced deficits on a lateralised choice reaction time task in rats (see also Chapter 3.3) as well as tests of drug induced rotations. In this study some of the simple motor tests proved to be sensitive to unilateral lesions. Therefore in the present experiment we chose to utilise some of the tests that were sensitive to detect lesion induced changes form experiments 4 and 5 (Chapter 3.4 and 3.5, respectively). Specifically, we directly investigated the restorative ability of motor function of mouse primary fetal tissue in the denervated striatum of 6-OHDA lesioned mice on simple and complex behavioural tests.

The experiment conducted in the present paper as well as analysis of the data, histology and preparation of the manuscript was undertaken entirely by myself. Professor S.B. Dunnett was involved in planning of the experiment and gave help and advice throughout as well as in the writing of the manuscript. Ms. Ngoc-Nga Vinh helped with the dissection of the embryos as well as with the preparations of the cell suspensions prior to transplantation.

EJN fens EUROPEAN JOURNAL OF NEUROSCIENCE

*European Journal of Neuroscience, Vol. 37, pp. 1691–1704, 2013* doi:10.1111/ejn.12150

DISORDERS OF THE NERVOUS SYSTEM

# Behavioural recovery on simple and complex tasks by means of cell replacement therapy in unilateral 6-hydroxydopamine-lesioned mice

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*Keywords*: choice reaction time, dopamine, graft, motor behaviour, Parkinson's disease, ventral mesencephalon

## Abstract

Before cell replacement therapies can enter the clinic, it is imperative to test the therapeutic benefits in well-described animal models. In the present study, we aimed to investigate the effects of 6-hydroxydopamine lesions to the medial forebrain bundle and subsequent grafting of embryonic day (E)12.5 ventral mesencephalon into the denervated striatum in C57/Bl6 mice on a battery of simple motor tests (drug-induced rotation, rotarod, and corridor) and the lateralised choice reaction time task conducted in the mouse nine-hole box. Histological analysis confirmed effective lesions and good graft survival. The lesion induced marked deficits in the choice reaction time task, the rotarod test, and corridor test, and these deficits were partially but significantly alleviated in the grafted mice. Although the lesions induced significant rotation following injections of amphetamine and apomorphine, respectively, the grafts did not, suprisingly, alleviate the rotation deficit. This study shows the ability of ventral mesencephalic tissue to ameliorate some of the lesion-induced deficits, and the power of operant testing in detecting small but significant improvements. The behavioural tests presented are useful drug-free approaches for evaluating cell-based therapies.

## Introduction

Parkinson's disease (PD) is a progressive, neurodegenerative disorder that is characterised by loss of dopaminergic cells in the ventral mesencephalon (VM) (Parkinson, 2002; Braak *et al.*, 2003; Björklund & Dunnett, 2007a,b; Lees *et al.*, 2008). For more than four decades, the standard model for PD and for characterisation of the effect of cell replacement therapies has been the unilateral 6-hydroxydopamine (6-OHDA) lesion to the medial forebrain bundle (MFB) in the rat (Ungerstedt, 1968; Ungerstedt & Arbuthnott, 1970; Kirik *et al.*, 1998; Grealish *et al.*, 2008). There has been a recent rise in interest in developing and characterising mouse lesion models of PD (Lundblad *et al.*, 2004, 2005; Cenci & Lundblad, 2007; Grealish *et al.*, 2010b; Heuer *et al.*, 2012a,b; Smith *et al.*, 2012a). These have the additional advantage over the rat that: (i) next to the lesion, additional neuropathology can be investigated, i.e. via transgenic lines (Fleming *et al.*, 2005; Harvey *et al.*, 2008); and (ii) genetic manipulations of cells are more easily achieved (Jaeger *et al.*, 2011).

Cell replacement therapy promises to be a valuable therapeutic tool for a subset of patients (Perlow *et al.*, 1979; Dunnett & Björklund, 1999; Björklund & Lindvall, 2000; Björklund *et al.*, 2003). With the advent of alternative cell sources to primary fetal tissue, it is imperative to compare the different cell types that are already used for grafting with respect to the functions that the graft can restore and the functions that it cannot restore. Basic tests of motor asymmetry, such as drug-induced rotations, are useful for screening dopamine depletion/release, but they do not resemble the complex loss of motor function that is seen in humans (Dowd & Dunnett, 2004). Although it has not hitherto been possible to analyse in rodents all of the features that are seen in humans, operant behavioural analysis allows for a more complex breakdown of the behavioural pattern that is induced by the lesion. Furthermore, it has been shown previously that tests of simple motor function may not be sensitive enough to detect small, but significant, therapeutic effects (Dowd & Dunnett, 2004). The lateralised choice reaction time task, originally designed by Carli *et al.* (Carli *et al.*, 1985, 1989), has been shown to be a powerful tool for the analysis of the underlying deficit of unilateral dopamine depletion (Brown & Robbins, 1991; Dowd & Dunnett, 2004, 2005a,b; Heuer & Dunnett, 2013) and the effects of cell replacement therapies in rats (Dowd & Dunnett, 2004; Heuer *et al.*, 2013). We recently adapted the lateralised choice reaction time task, first developed for rats, for mice, and characterised the effects of dopamine depletion in C57/Bl6 mice (Heuer *et al.*, 2012a). Thus far, grafting in mice has been performed with limited success, and behavioural analysis has largely focused on drug-induced tests (Low *et al.*, 1987; Shimizu *et al.*, 1988; Thompson *et al.*, 2009; Smith *et al.*, 2012b); from our own experience, with all experimental factors being equal (dissector, media, grafting procedure, etc.), mouse grafts have been

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*Received 24 November 2012, revised 7 January 2013, accepted 9 January 2013*

smaller and less reliable than rat grafts, with many grafts showing 'pencil-line' morphology.

Here, we analysed the effects of primary fetal [embryonic day (E)12.5] VM single-cell suspension grafts on behavioural recovery on a series of simple motor tests and the lateralised choice reaction time task in mice that received unilateral 6-OHDA lesions to the MFB.

## Materials and methods

### Subjects

Thirty-eight male mice of the C57/Bl6 strain (Charles Rivers, Margate, Kent, UK) were used in this experiment. All mice were housed in pairs in standard laboratory cages under a 10-h:14-h dark/light cycle (lights on at 06:00 h) at 21 ± 2 °C. All mice had free access to water at all times, but the amount of food was reduced during the experimental phases, to restrict their body weight to 90% of the free-feeding weight. Weighed amounts (2–4 g per mouse) of laboratory chow were placed directly into the home cages approximately 1 h after the testing session. This experiment was conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 and local ethical review at Cardiff University.

### Experimental plan

After pre-training, mice were allocated to three equal groups matched for response accuracy. Two groups (lesion and graft) received unilateral lesions via infusion of 6-OHDA into the MFB. Three weeks post-lesion, we assessed the effect of the lesion on the choice reaction time task and on tests of simple motor behaviour (corridor, rotarod, and rotations). Once testing was concluded, one lesion group was grafted with a single-cell suspension of dopamine-rich tissue, implanted into the denervated striatum. The mice were rested for 12 weeks to allow the grafts to mature, following which they were re-tested on the choice reaction time task for two consecutive weeks and one additional week in a probe trial configuration. Once operant testing was concluded, post-graft performance was assessed on the tests of simple motor function before the mice were killed and their brains were processed.

### Operant chambers

Testing was conducted in a bank of 16 operant nine-hole chambers (Campden Instruments, Loughborough, UK) that were controlled by a desktop computer running BNC software under the Windows XP operating system. The dimensions of each chamber were 14 × 13.5 × 12.5 cm (Humby *et al.*, 2005; Heuer *et al.*, 2012a). On one of the walls, a curved horizontal array was fitted, with nine response holes. Each hole had a photocell detector to detect entries into the hole (i.e. nose-pokes) and an LED stimulus light. In the present experiment, only three of the response holes were used (holes 3, 5, and 7), and the remaining unused holes were blocked. On the opposite wall, a food magazine was fitted, into which 5 μL of strawberry milk (Yazoo, Campina, UK) could be delivered via a peristaltic pump. An infrared beam at the entrance of the magazine detected entries by the mice when they collected the reward. The magazine was fitted with a panel light, which was used to indicate delivery of a reward, and the chamber was fitted with two additional 'house' lights on the side walls, which were used to signal procedural errors. Each chamber was fitted in a sound-attenuating cubicle with an air extractor fan.

### Behavioural tasks

#### *Operant training*

Behavioural testing was started 1 week after the introduction of the food restriction regime. Training for the operant task outlined below began with a series of shaping days. On the first day, the mouse was introduced to the test chamber with the magazine light on, and a 25-μL reward was delivered into the magazine. When the mouse had collected the reward, an inter-trial interval of 10 s was started, with the house lights switched on and the magazine light extinguished. After the inter-trial time had elapsed, a 5-μL reward was delivered into the magazine, and the house lights were switched off and the magazine light illuminated. This initial shaping session lasted for 15 min. For the next 3 days of training, the chamber was dark, with only the centre hole (hole 5 on the array) illuminated. A nose-poke into the illuminated hole resulted in the centre hole light being switched off, and a 5-μL reward was simultaneously delivered into the illuminated magazine. After reward collection, the magazine light was switched off, and the centre hole light was illuminated again. On the first day of training, the centre holes were baited occasionally to encourage the mice to nose-poke. After 4 days, all mice were poking into the centre hole, and progressed to the training phase of the lateralised choice reaction time task.

#### *Lateralised choice reaction time task*

The lateralised reaction time task has been described in detail for the rat (Carli *et al.*, 1985; Dowd & Dunnett, 2004), and recently for the mouse (Heuer *et al.*, 2012a). In brief, each trial started with the chamber darkened and the centre hole (hole 5) lit. When the mouse responded with a sustained nose-poke into the illuminated centre hole, the light was switched off, and one of the lateralised holes (hole 3 or hole 7) was briefly lit (stimulus duration). When the mouse responded correctly by nose-poking into that hole, it received a 5-μL reward into the illuminated magazine. Upon reward collection, the magazine light was extinguished and the inter-trial interval started, with the house light being illuminated. When the mouse responded into the incorrect hole, it was 'punished' by a time-out period in darkness. During the initial training phase, the stimulus duration was reduced from 10 s to 300 ms, and the period over which the mouse was required to hold its initial nose-poke was increased progressively from 10 ms to 100 ms. After all mice had reached a stable level of performance, baseline data were collected with the following parameters: holds – 25, 50, 75 or 100 ms; stimulus duration – 300 ms; inter-trial interval – 2 s; time-out – 5 s; and session length – 30 min.

The outcome measures analysed in the present task were: the total number of trials usable (the number of trials in which the mouse sustained its nose-poke for the required delay); response accuracy (the number of correct responses divided by the number of trials usable, and expressed as a percentage); incorrect trials (the number of incorrect responses divided by the number of usable trials, expressed as a percentage); reaction time (the latency from presentation of the lateralised stimulus light to withdrawal from the centre hole); and movement time (the latency from withdrawal from the centre hole to the response in the lateralised response location).

#### *Simple behavioural screens*

As operant analysis of behaviour is not widely performed in the lesion and graft model in mice, we chose to compare the behavioural effects of the lesion and the graft on a number of simple conventional motor tests that have previously been shown to be sen-

sitive to unilateral dopaminergic depletion. Whether these tasks are sensitive in detecting changes of graft-induced recovery is unknown at this time, and a direct comparison is therefore warranted. We tested all mice on a series of tests, which are outlined below. All tests were conducted after completion of operant testing, while the mice were still on food restriction for the corridor test, and when they had been free-feeding for at least 3 days for all other tests.

#### *Drug-induced rotation*

Drug-induced rotations were conducted in automated rotometer bowls, which were modelled after the design of Ungerstedt (Ungerstedt & Arbuthnott, 1970). All mice were challenged 3 weeks post-lesion with 2.5 mg/kg intraperitoneal methamphetamine, and post-graft after all behavioural data had been gathered with the same dose of methamphetamine and with 0.01 mg/kg subcutaneous apomorphine 5 days after the last amphetamine challenge. Both drugs were dissolved in 0.09% sterile saline solution as carrier vehicle, and prepared fresh on the day of testing. Rotation scores were expressed as the average of all net ipsilateral rotations when mice were injected with amphetamine [∑(clockwise turns – counterclockwise turns)/min], and as the average of all net contralateral rotations when mice were injected with apomorphine [∑(counterclockwise turns – clockwise turns)/min].

#### *Rotarod*

Overall performance in motor coordination, balance and strength was assessed on the rotarod apparatus (Ugo Basile, Varese, Italy). Mice were trained on a fixed-speed protocol, at 4, 12 and 24 r.p.m. for 300 s on three separate occasions (on separate days) (Heuer *et al.*, 2012b). When mice fell off the rod during the training phase, they were placed back on until the full time had elapsed. On the testing day, the mice were tested three times, on the accelerating version of the rotarod, which increased the speed of rotation from 4 to 44 r.p.m. over a period of 300 s. Mice were given a break of at least 1 h between runs. The average of the best two runs was taken as the outcome measure.

#### *Corridor*

Lateralised sensorimotor proprioception and neglect were assessed with the corridor test, which was recently adapted from the rat for use in mice (Grealish *et al.*, 2010b). Mice were habituated to the apparatus for two consecutive days. They were placed into the corridor for 10 min on each day, with some 10-mg sucrose pellets scattered around the corridor floor. For the testing, 10 pairs of adjacent pots were filled with five 10-mg precision sugar pellets along the walls of the corridor [see Grealish *et al.* (2010b) for details]. Retrievals were scored as investigations into a pot, regardless of whether the mouse actually took a pellet or not. On the test day, all mice were habituated to the corridor for 5 min in one compartment, and then transferred to the baited testing corridor (Grealish *et al.*, 2010b). They were removed after either they had made 20 explorations/retrievals or 5 min had elapsed. Data were expressed as a percentage of contralateral retrievals.

## Surgery

### *Lesion surgery*

The mouse was prepared for surgery by induction of anaesthesia, shaving the surgery area, and securing the head in a stereotaxic frame. Anaesthesia was maintained with 1.5–2.0% isoflurane anaesthetic in a 2 : 1 oxygen/nitrous oxide mixture. Lesions were aimed at the MFB at the following coordinates (from bregma): AP, −1.2 mm, ML, −1.2 mm; and DV, −4.75 mm (from dura); the nose-bar was set at 0.00 mm. A volume of 1.5 μL of 6 μg/μL 6-OHDA (hydrobromide salt, calculated from free base weight), dissolved in a solution of 0.9% sterile saline and 0.2 mg/mL ascorbic acid, was injected. Injections were made via a 30-gauge stainless steel cannula connected via polyethylene tubing to a 10-μL Hamilton syringe, which was driven by an automatic micro-drive pump. After infusion of the toxin over a period of 3 min, the injection needle was left in place for an additional 3 min to allow for diffusion, before the needle was carefully retracted and the wound was sutured. All mice were given 0.15 μL of Metacam (Meloxicam; Boehringer, Ingelheim, Germany) as an analgesic, and 1 mL of glucose (4%) saline (0.09%) solution post-lesion. For 10 consecutive days following lesion surgery, all mice received daily glucose/saline injections and were provided with wet palatable food in their home cages to prevent dehydration (Heuer *et al.*, 2012b).

### *Graft surgery*

Graft tissue was harvested from three pregnant C57/Bl6 dams at E12.5. The quasi-single-cell suspension was prepared according to a standard protocol (Mayer *et al.*, 1992). In brief, dissected VMs were washed in Dulbecco's Modified Eagle's Medium (DMEM/F12; Life Technologies, Paisley, UK) before being treated with 0.01% trypsin (Trypsin; Worthington, Lakewood, NJ, USA)/DNase (DNAse; Sigma, Dorset, UK) for 10 min at 37 °C. Tissue was then washed with DMEM/F12, and collected by centrifugation at 180 ***g*** for 3 min. The medium was aspirated, cells were re-suspended in 200 μL of fresh DMEM/F12 at a density of 300 000 cells/μL, and 1 μL was injected into the denervated striatum at each of the following coordinates (mm) from Bregma: AP, +0.8; ML, −1.7; and DV, −3.0 and −2.8; the incisor bar was set to 0 mm relative to the interaural line (total, 600 000 cells; 2 × 1 μL of 300 000). The grafting cannula was left in place for an additional 3 min to allow for diffusion of the suspension before slow retraction of the grafting cannula. Post-operative care was as described above.

## Histology

### *Immunohistology*

After behavioural testing was completed, all mice were deeply anaesthetised with 200 mg/kg sodium pentobarbitone and transcardially perfused with 25 mL of phosphate-buffered saline (pH 7.3) as a prewash, followed by 50 mL of 1.5% paraformaldehyde in phosphate-buffered saline. After the brains had been carefully removed from the skull, they were post-fixed for an additional 24 h before being cryoprotected via immersion in a 25% sucrose solution, where they were kept until they had sunk. Thereafter, all brains were cut on a freezing sledge microtome into coronal sections at a thickness of 40 μm. Cut sections were then stored at +4 °C in Tris-buffered saline (pH 7.4) with 2% azide. A 1 : 6 series of sections for each brain was stained according to standard immunohistochemical protocols (Torres & Dunnett, 2007; Heuer *et al.*, 2012a,b) for tyrosine hydroxylase (TH) with a rabbit TH primary antibody (polyclonal; Chemicon; 1 : 1000). Stained sections were mounted on glass slides, air-dried overnight, dehydrated in an ascending series of alcohols and xylene, and coverslipped with a DPX mounting medium.

### *TH-immunoreactive (TH-ir) cell counts*

Cells that stained positive for TH in the ipsilateral and contralateral substantia nigra (SN) and ventral tegmental area (VTA) were counted at the level of the medial terminal nucleus of the accessory nucleus of the optic nerve. TH-ir cells were counted under ×10 magnification with a Leica DM/RBE microscope, and the counts were expressed as percentage of contralateral.

### *Analysis of graft survival*

All cells staining positively for TH were counted on an Olympic C.A.S.T. grid system in a 1 : 6 series of sections. One hundred cells were randomly selected over 10 grafts, and their average diameter was measured. The total number of cells within the graft was estimated with Abercrombie's (Abercrombie, 1946) correction procedure:

$$T = F \times A \times M/(D/M)$$

where $T$ is the total number of cells, $F$ is the frequency of sections, $A$ is the total count, $M$ is the section thickness, and $D$ is the average cell diameter.

### *Statistics*

All data were analysed with the GENSTAT v13.1 software package (VSN International Ltd., Hemel Hempstead, UK; V12.1.0) with the significance level set at $\alpha = 0.05$ for significant $F$-ratios. Collated data from the operant testing were analysed with repeated measures ANOVA with the factors group (control, lesion, and graft), side (ipsilateral and contralateral), and week (baseline, post-lesion, post-graft 1, and post-graft 2). Averages of reaction and movement time latencies from the operant test were expressed as geometric, rather than arithmetic, means, to reduce the influence of extreme data points. *Post hoc* analysis was conducted with Student–Newman–Keuls test, as appropriate.

## Results

### *Histology results*

After inspection of the stained sections, TH-ir cells were counted in the SN and VTA to estimate the magnitude of the lesions, and in the striatum to estimate cell numbers in the graft. Mice that had > 30% cells remaining on the lesioned side ($n = 6$) or no surviving cells in the graft ($n = 3$) were excluded, resulting in final group sizes for the statistical analysis of eight controls, 11 lesioned mice, and 10 grafted mice.

Infusion of 6-OHDA into the MFB caused degeneration of TH-ir cells in the VM. As shown in Fig. 1, mice of both lesion groups had a reduced number of TH-ir cells in the ipsilateral SN (group, $F_{2,27} = 190.85$, $P < 0.001$). Both lesion groups had an approximately 89% reduction in ipsilateral SN cell counts, which differed significantly from the control group ($P < 0.001$). Furthermore, all lesioned mice had a significant reduction in the number of TH-ir cells in the ipsilateral VTA (group, $F_{2,27} = 20.85$, $P < 0.001$), with both lesion groups showing a similar level of depletion as compared with controls ($P < 0.001$). After the grafting of primary fetal VM into the denervated striatum, viable TH-ir cells (1649 ± 342) could readily be detected at the transplantation site.

### *Assessment on the lateralised choice reaction time task*

#### *Trials attempted*

During baseline assessment, all mice produced a high number of usable trials by responding to the illumination of the centre hole by a sustained nose-poke for the required delay [Fig. 2A; group, $F_{2,27} = 0.84$, not significant (NS)]. After the lesion, mice of the control group continued to produce a high number of usable trials, whereas mice of both lesion groups produced usable trials of between 20% and 25% of their baseline performance (group, $F_{2,27} = 11.32$, $P < 0.001$). At this block of testing, there was no difference between the lesion groups in the number of usable trials produced ($t = 0.66$, NS), with both lesion groups producing significantly fewer trials than the control group (both, $t > 8.32$, $P < 0.01$). Engraftment of dopamine-rich tissue did not have an effect on the number of usable trials, as the difference between the groups was not different per block of testing ($F_{2,27} = 0.61$, NS). Newman–Keuls *post hoc* tests revealed that there were no differences between the graft and the lesion groups at either of the post-graft blocks of testing (both, $t < 1.84$, NS).

#### *Error results*

The types of procedural error recorded in the choice reaction time task were: (i) premature withdrawal of the sustained nose-poke before the delay period had elapsed (ii) perseverative centre hole response; and (iii) omission of lateralised response. All errors per week of testing are presented in Table 1. During baseline testing, there was no difference between the three groups on any of the procedural errors (all, $F_{1,27} \leq 1.95$, NS). The number of premature withdrawals increased with the weeks of testing (week, $F_{3,81} = 3.82$, $P < 0.05$), but was not different between the three experimental groups (week × group, $F_{6,81} = 0.96$, NS). The number of repeated centre pokes increased post-lesion and post-graft, but the difference failed to reach statistical significance (week × group, $F_{6,81} = 2.18$, $P = 0.053$). Overall, there was a significant main effect of week (week, $F_{3,81} = 7.12$, $P < 0.001$) and a significant difference between the groups (group, $F_{2,27} = 3.92$, $P < 0.05$). As compared with the control group, whose repeated centre pokes accounted for < 10% of errors, both lesion groups produced, on average, > 60% repeated centre pokes at the post-lesion block of testing (Table 1). The lack of a significant interaction effect is most likely attributable to the large variation in the data. The largest proportion of errors made in the present task was omission of the lateralised response. Whereas mice of all groups initiated the trials via the centre poke, post-lesion > 75% of all trials were not completed by the mice that received unilateral 6-OHDA lesions (week × group, $F_{6,81} = 4.89$, $P < 0.001$). At both post-graft blocks of testing, mice that received grafts omitted fewer responses than mice of the lesion group, and, at the last time point of testing, they were not significantly different from controls.

#### *Accuracy results*

Response accuracy was calculated as the number of correct responses divided by the number of usable trials, and expressed as a percentage. During assessment of baseline performance, mice of all groups responded accurately to the lateralised stimulus (> 90% correct), with no difference between the groups (Fig. 2B; group, $F_{2,27} = 2.17$, NS). Furthermore, there was no difference between the sides of stimulus presentation in response accuracy (side, $F_{1,27} = 0.75$, NS).

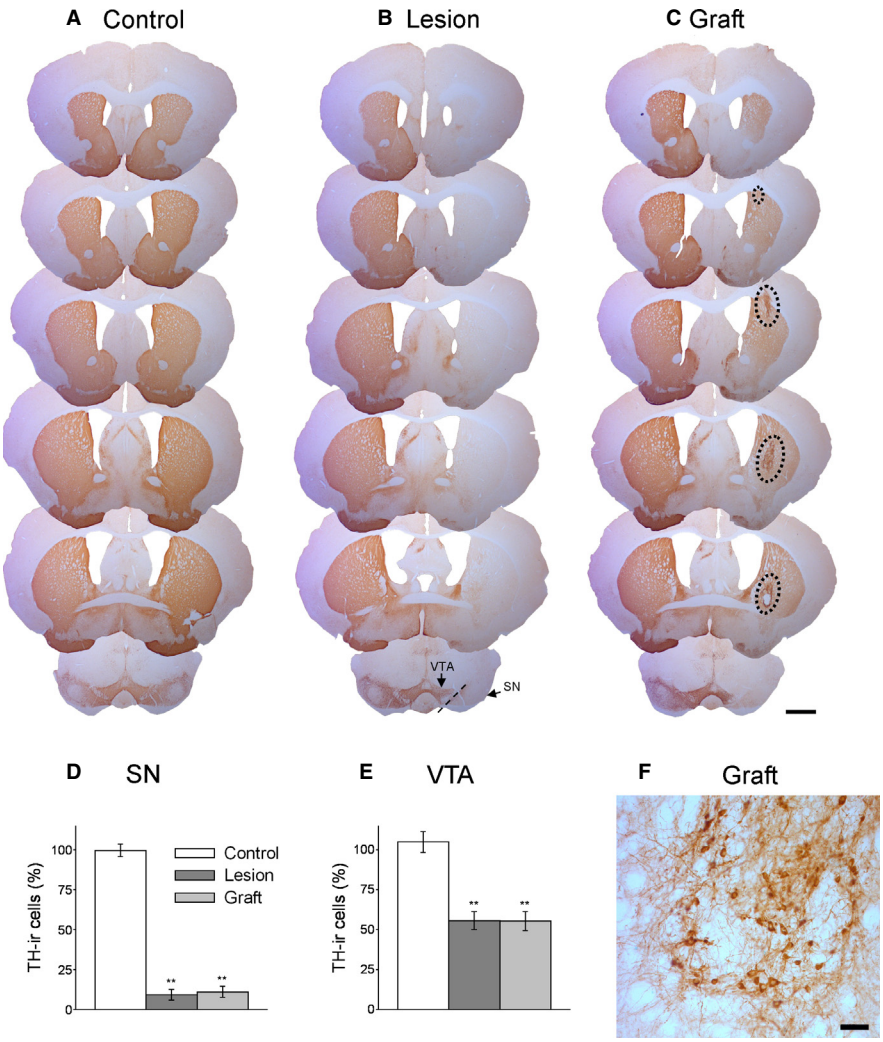


FIG. 1. Representative series of TH-stained sections for control (A), lesioned (B) and grafted (C) mice, respectively. The dashed line in B indicates the separation of the VM into the VTA and SN, and the dotted line in C indicate the location of the graft in the respective section. Scale bar: 1000 μm. (D and E) Cell numbers of TH-stained sections at the level of the VM for cells in the SN (D) and the VTA (E), respectively. Asterisks denote significant difference from control at the $P < 0.001$ level of significance. (F) High magnification (×20) of the grafted TH-ir cells. Scale bar: 50 μm.

After this highly accurate performance during baseline training, the lesion caused a significant reduction in response accuracy (group, $F_{2,27} = 16.01$, $P < 0.001$). Both lesion groups performed with a significantly reduced response accuracy as compared with the control group (all, $t > 11.49$, $P < 0.01$), whereas there was no difference in performance between the experimental groups ($t < 0.15$, NS), as they were matched according to this parameter. Although responses to both sides were reduced as compared with baseline testing (Fig. 3A; week × side × group, $F_{6,81} = 3.16$, $P < 0.01$), responses made to contralateral stimuli were less frequent than responses made to ipsilateral stimuli (side, $F_{1,27} = 13.88$, $P < 0.01$).

During the first week of testing after the grafting, there was still a difference in overall response accuracy between the groups (group, $F_{2,27} = 12.01$, $P < 0.001$), with the control group performing significantly better than the two experimental groups (all, $t > 8.41$, $P < 0.01$).

Although not significantly different from controls, grafted mice showed better overall accuracy scores and performed significantly better than mice of the lesion-only group ($t_{28} = 4.59$, $P < 0.05$).

As during post-lesion assessment, mice that were lesioned performed worse on the side contralateral to the lesion (side × group, $F_{2,27} = 4.81$, $P < 0.05$). *Post hoc* testing on the contralateral side revealed that the graft group responded with a higher response accuracy than the lesion-only group ($t_{28} = 7.59$, $P < 0.01$). Analysis of ipsilateral performance revealed that both lesion groups performed, as previously, with a reduced response accuracy as compared with the control group (all, $t_{28} > 9.68$, $P < 0.01$), but there was no difference between the graft and lesion-only groups at this time point of testing (both, $t_{28} < 0.67$, NS).

There was no change in response accuracy between the two blocks of post-graft testing (week × group, $F_{1,27} = 3.10$, NS), with the effects for group, side and the interaction between them being similar to those during the first week of post-graft assessment. At this time point, all lesion groups performed with a lower overall response accuracy than the control group (both, $t_{28} > 3.53$, $P < 0.05$). Similarly to the first week post-graft assessment, mice that received grafts responded with higher overall response accuracy than their lesion-only counterparts ($t_{28} = 5.44$, $P < 0.01$). Whereas there was no difference in ipsilateral accuracy between the lesion and graft groups after transplantation (both, $t_{28} < 1.53$), contralateral accuracy was significantly higher for the graft group at both time points (both, $t_{28} > 7.59$, $P < 0.01$).

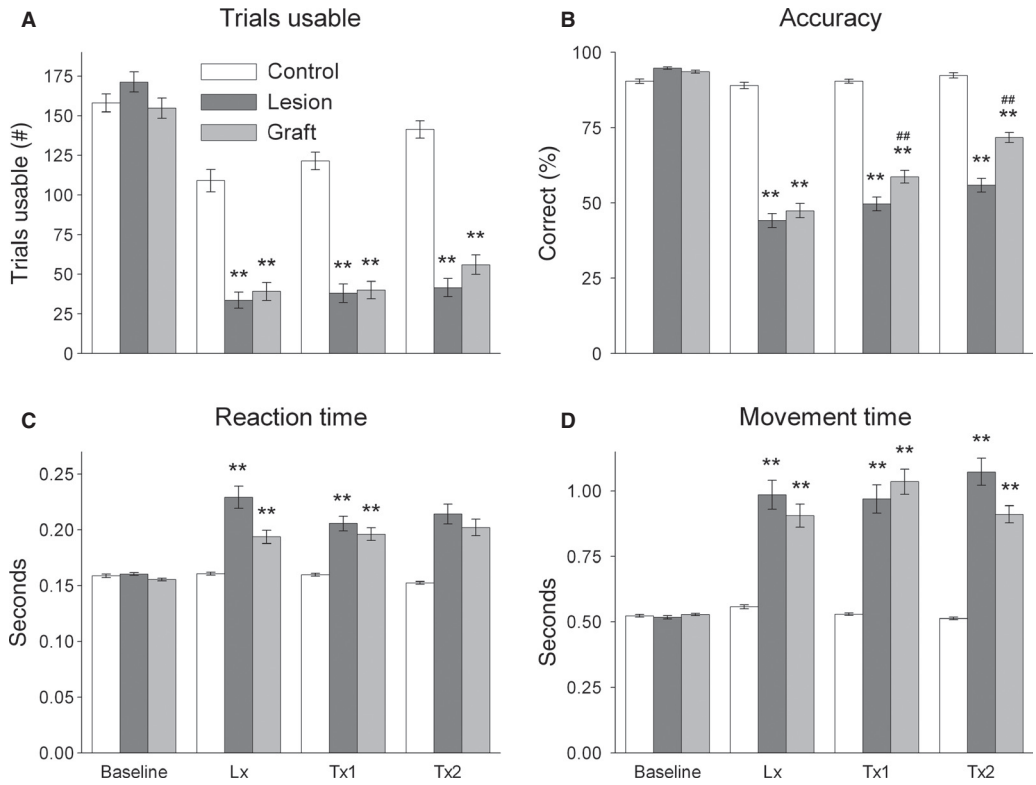


FIG. 2. Main effects of the lateralised choice reaction time task by week of testing. Data were collated over 5 days per block at baseline, at post-lesion (Lx), and at the first (Tx1) and second (Tx2) weeks of post-graft testing. (A) Total number of usable trials in which the mouse sustained the nose-poke for the required delay and executed a lateralised response. (B) Percentage accurate responses on the total number of usable trials. (C) Reaction time latencies on correct trials. (D) Movement time latencies on correct trials. The number of symbols above the bars denotes significant differences at the $P < 0.05$ (for one symbol) and $P < 0.01$ (for two symbols) levels of significance. Asterisks denote significant differences from the control group, and hashes denote significant differences from the lesion group, as determined by Student–Neuman–Keuls *post hoc* analysis.

In summary, the data show that the lesion causes a reduction in overall response accuracy, with the side contralateral to the lesion being the more impaired. After the grafting, all three groups improved in contralateral responding, but only the group that was grafted with primary fetal VM improved when a response had to be made towards a contralateral stimulus, while still being impaired as compared with mice of the control group.

*Percentage incorrect trials*

The percentage of incorrect responses (data not shown) was different between the three groups (group, $F_{2,27} = 6.30$, $P < 0.01$), and increased over the weeks of testing (week × group, $F_{6,81} = 3.56$, $P < 0.01$). During baseline testing, across all three groups the percentage of incorrect responses (responses to the incorrect hole) was 5.22% ± 0.28%. After the lesion, mice of the control group continued to respond to the incorrect hole at a frequency of 6–7% at all three time points. Mice of the lesion groups showed an increased number of incorrect responses, and responded to the incorrect hole on 12–20% of all usable trials. Incorrect responses were higher for both lesion groups when the stimulus was presented on the contralateral side to the lesion (week × side × group, $F_{6,81} = 3.50$, $P < 0.01$). For mice of the lesion group, incorrect responses to contralateral stimuli constituted 22.5, 23 and 35% of all usable trials, and incorrect responses to ipsilateral stimuli constituted 13, 5, and 5%, when an ipsilateral stimulus was presented across the three post-lesion time points. Mice that were grafted showed a similar response pattern, with 16, 18 and 21% incorrect responses towards a contralateral stimulus, and with 8, 15 and 12% incorrect responses towards an ipsilateral stimulus, for each of the three post-lesion time points, respectively. The data clearly show that the deficit in response accuracy can be broken down into three parts: an omission of the lateralised response, a repeated centre poke (Table 1), or a response into the incorrect hole. Mice of both lesion groups showed a bias for responding to the hole ipsilateral to the lesion.

*Reaction and movement time latencies*

There was no difference between the groups during assessment of baseline performance on either reaction time (Fig. 2C; group, $F_{2,27} = 0.44$, NS) or movement time (Fig. 2D; group, $F_{2,27} = 0.10$, NS). All mice reacted and responded to stimuli to either side with similar latencies (both, side, $F_{2,27} < 0.01$, NS).

After the lesion, reaction times were different between the groups (group, $F_{2,27} = 5.31$, $P < 0.05$), with both lesion groups reacting more slowly to the lateralised stimulus light than the control group (both, $t > 4.56$, $P < 0.01$). The lesion-only group reacted significantly more slowly than the graft group before grafting ($t_{28} = 3.12$, $P < 0.05$). As reported previously (Heuer *et al.*, 2012a), reaction times were not differently affected according to the side of stimulus presentation (Fig. 3B; side, $F_{1,27} = 0.53$, NS).

The movement time latencies to execute the lateralised response were different between the three groups after the lesion (group, $F_{1,27} = 6.35$, $P < 0.01$). Both lesion groups took longer to execute the response than the control group (both, $t > 5.07$, $P < 0.01$), whereas there was no significant difference in movement time latency between the two lesion groups ($t_{28} = 2.22$, NS). In contrast to reaction times, the time taken to respond to the lateralised

TABLE 1. Procedural errors on the choice reaction time task

| | Control | Lesion | Graft | $F_{2,27}$ | $P$ |
|---|---|---|---|---|---|
| Premature withdrawal (%) | | | | | |
| Baseline | 8.91 ± 0.48 | 7.51 ± 0.47 | 10.16 ± 0.79 | 1.95 | NS |
| Lx | 9.80 ± 0.60 | 10.47 ± 1.01 | 13.00 ± 1.18 | 1.74 | NS |
| Tx1 | 9.82 ± 0.56 | 10.44 ± 0.96 | 10.88 ± 1.01 | 0.12 | NS |
| Tx2 | 9.98 ± 0.70 | 13.73 ± 0.96 | 12.52 ± 1.01 | 0.16 | NS |
| Perseverative centre poke (%) | | | | | |
| Baseline | 7.13 ± 0.54 | 7.20 ± 1.40 | 6.18 ± 0.56 | 0.21 | NS |
| Lx | 9.78 ± 1.80 | **61.31 ± 18.00**** | **61.06 ± 9.03**** | 4.19 | < 0.05 |
| Tx1 | 8.00 ± 0.62 | **47.15 ± 10.69*** | **39.48 ± 7.72*** | 4.21 | < 0.05 |
| Tx2 | 6.87 ± 1.41 | 80.18 ± 22.21 | 38.26 ± 6.97 | 2.48 | NS |
| Omission (%) | | | | | |
| Baseline | 0.78 ± 0.10 | 0.76 ± 0.30 | 0.82 ± 0.19 | 0.01 | NS |
| Lx | 4.93 ± 3.70 | **77.15 ± 9.91**** | **79.41 ± 12.30**** | 5.72 | < 0.01 |
| Tx1 | 0.82 ± 0.24 | **77.55 ± 10.24**** | **35.94 ± 5.73**$^{**/\dagger\dagger}$ | 7.12 | < 0.01 |
| Tx2 | 0.38 ± 0.27 | **47.20 ± 8.74**** | **17.46 ± 4.00**$^{\dagger}$ | 5.84 | < 0.01 |

Lx, post-lesion; Tx1, first week of post-graft testing; Tx2, second week of post-graft testing; NS, not significant. Statistical analysis has been restricted to the individual weeks, as the interaction effect (week × group) had only borderline significance ($P = 0.053$). Significant group effects are presented per week of testing. Bold numbers denote significantly different groups, with the number of symbols denoting differences at the $P < 0.05$ (for one symbol) and $P < 0.01$ (for two symbols) levels, respectively. *Significantly different from control. †Significantly different from lesion.

response location was differently affected according to the side on which the response had to be executed (Fig. 3C; side × group, $F_{2,27} = 4.10$, $P < 0.05$). Overall responses towards the ipsilateral side of stimulus presentation were faster than responses towards the contralateral side (side, $F_{1,27} = 7.49$, $P < 0.05$; side × group, $F_{1,27} = 4.10$, $P < 0.05$).

Both lesion groups responded with increased movement time latencies towards the ipsilateral response location, as compared with the control group (both, $t_{28} > 2.93$, $P < 0.05$), whereas there was no difference in movement time between the two lesion groups ($t_{28} = 0.85$, NS) on this side of testing. On the contralateral side to the lesion, response times were also significantly longer for both lesion groups than for the control group (both, $t_{28} > 7.15$, $P < 0.01$). Furthermore, the lesion-only group was slower to execute the contralateral response than the graft group ($t_{28} = 4.29$, $P < 0.01$). The graft had no effect on reaction time performance, as there was a difference in reaction time latencies at the first block of post-lesion testing (group, $F_{2,27} = 4.40$, $P < 0.05$), with both lesion groups responding more slowly than the control group (both, $t > 3.86$, $P < 0.01$). During the last week of post-graft testing, the difference in reaction time latency between the groups failed to reach statistical significance (group, $F_{2,27} = 2.00$, NS).

Movement time latencies were differently affected between the groups post-graft (group, $F_{2,27} = 5.96$, $P < 0.01$) and were different between the sides of testing (side, $F_{1,27} = 8.22$, $P < 0.01$). The interaction effect between sides and groups failed to reach statistical significance (side × group, $F_{2,27} = 2.77$, $P = 0.083$). However, overall movement time latencies were slower for both lesion groups than for the control group (both, $t_{28} > 5.34$, $P < 0.01$). There was no difference between the lesion and the graft groups post-graft ($t_{28} = 0.58$, NS). Also, at the second week of post-graft testing, the effects of movement time were similar to those in the first week, with a difference in response times between groups (group, $F_{2,27} = 13.36$, $P < 0.001$), and both lesion groups taking longer to respond (both, $t > 3.50$, $P < 0.05$). Although grafted mice responded marginally faster than mice of the lesion group, this difference did not return significant from the *post hoc* analysis ($t_{28} = 1.43$, NS). Movement times during the second week post-graft were still slower when responses had to be made on the side contralateral to the lesion (side × group, $F_{2,27} = 6.17$, $P < 0.01$). Ipsilateral responses were slower for both lesion groups than for the control group (all, $t_{28} > 3.74$, $P < 0.05$), and the lesion group responded significantly more slowly than the graft group ($t_{28} = 3.00$, $P < 0.05$). For contralateral movements, the lesion groups responded significantly more slowly than the control group (both, $t_{28} > 8.08$, $P < 0.01$), with the lesion group responding more slowly than the graft group ($t_{28} = 3.34$, $P < 0.05$).

### *Probe trials*

During the last week of testing, we increased the duration of the lateralised stimulus light to 5 s (the length of the limited response time), which was longer than the animals' movement time during the standard configuration. We assumed that this would decrease task demand, as the mice could use the light as a beacon to guide their responses to the correct location.

Although all mice produced more usable trials than during testing with the standard configuration, there was still a difference in the number of usable trials produced (Fig. 4A; group, $F_{2,27} = 16.16$, $P < 0.001$). Both lesion groups produced significantly fewer trials than the control group (all, $t_{28} > 5.63$, $P < 0.01$). Although, as during standard testing, grafted mice produced a higher number of usable trials than the lesion groups, this difference did not reach statistical significance during *post hoc* testing ($t_{28} = 2.78$, NS).

Response accuracy was also different during probe testing (Fig. 4B; group, $F_{2,27} = 5.80$, $P < 0.01$), with, interestingly, both the control group and the graft group performing with similar accuracy ($t = 0.62$, NS), and both groups differing significantly from the lesion group (both, $t > 3.95$, $P < 0.01$). Response accuracy was, as previously, affected differently, depending on the side of stimulus presentation (Fig. 5A; side × group, $F_{2,27} = 6.34$, $P < 0.01$). Whereas there were no significant differences on the ipsilateral side of testing between any of the three groups (all, $t_{28} < 1.92$), response accuracy on the contralateral location was significantly impaired for the lesion group as compared with the control group and the graft group (both, $t_{28} > 5.31$, $P < 0.01$). Interestingly, the graft group did not differ significantly in

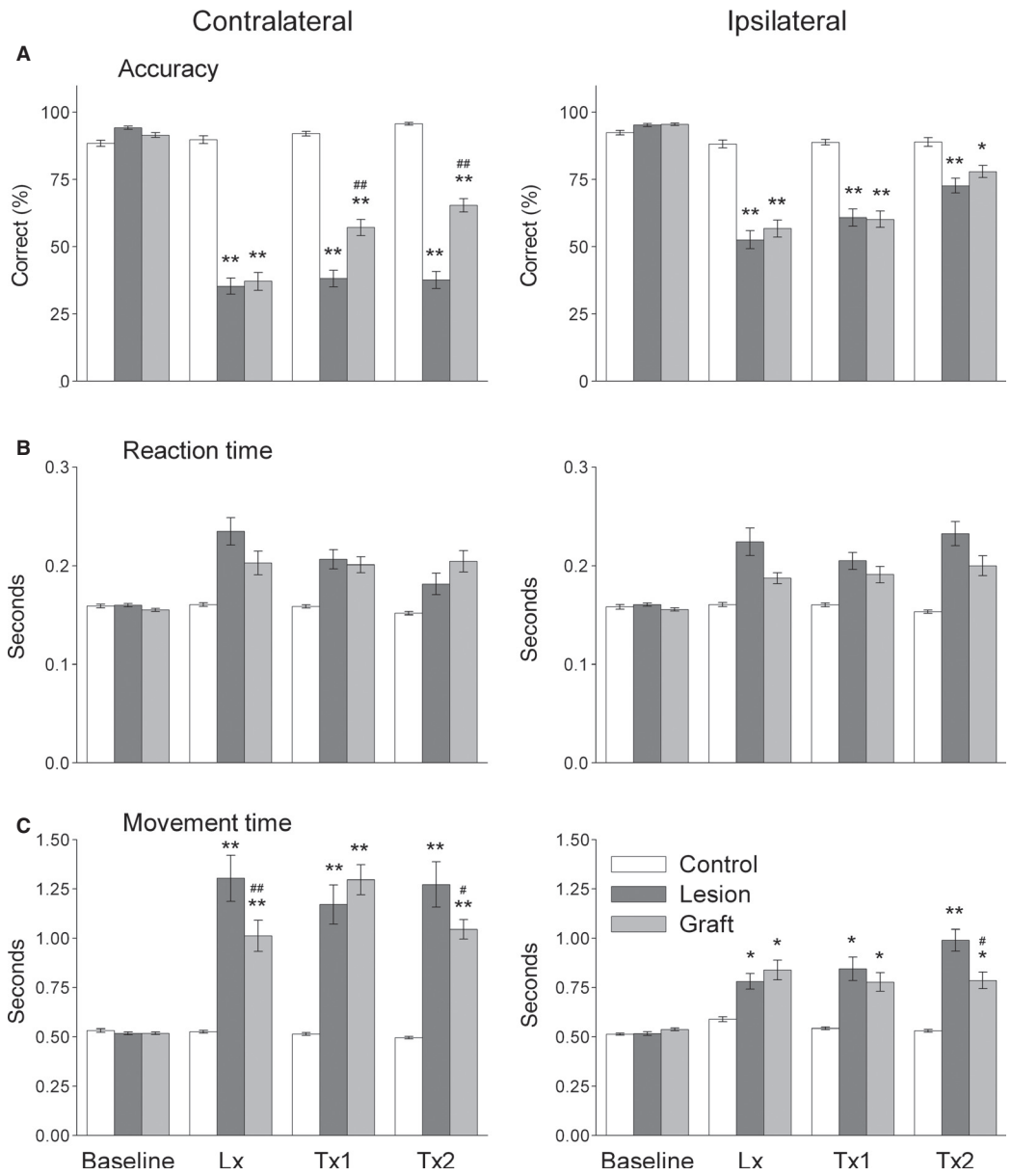


FIG. 3. Main effects on the lateralised choice reaction time task by side of response for contralateral and ipsilateral responses, respectively. Data were collated over 5 days per block at baseline, at post-lesion (Lx), and at the first (Tx1) and second (Tx2) weeks of post-graft testing. Note that simple effects are only analysed when significant interactions have been found in the ANOVA; that is, although differences in reaction time seem to be lateralised in the graph, the overall ANOVA did not show a significant week × group × side interaction. The number of symbols denotes significant differences at the $P < 0.05$ (for one symbol) and $P < 0.01$ (for two symbols) levels of significance for *post hoc* tests conducted after significant main effects were reported. Asterisks denote significant differences from the control group, and hashes denote significant differences from the lesion group. The task parameters analysed were: (A) response accuracy on correct trials (B) reaction time on correct trials; and (C) movement time latencies on correct trials.

contralateral response accuracy from the control group ($t_{28} = 1.87$, NS). As previously, reaction time performance was not significantly different between any of the three groups (Figs 4C and 5B; group, $F_{2,27} = 2.37$, NS). In contrast to reaction time, the time taken to execute the lateralised response was differently affected between the groups (Fig. 4D; group, $F_{2,27} = 13.52$, $P < 0.001$). Whereas the graft group did not respond significantly more slowly than the control group ($t_{28} = 2.68$, NS), the lesion group showed overall increased movement times as compared with the control group ($t > 5.43$, $P < 0.01$), but not as compared with the graft group ($t_{28} = 2.75$, NS). The deficit in movement time was lateralised, with responding to the side contralateral to the lesion being more impaired (Fig. 5C; side × group, $F_{2,27} = 11.16$, $P < 0.001$). When the response had to be directed towards the side ipsilateral to the lesion, there was no significant difference in movement time latencies between the groups (all, $t_{28} < 1.47$, NS). When a contralateral response was required, both lesion groups responded more slowly than the control group (both, $t_{28} > 3.47$, $P < 0.05$), and the graft group was less impaired than the lesion group ($t_{28} = 7.43$, $P < 0.01$).

### *Simple behavioural screen results*

#### *Rotations*

*Amphetamine-induced rotations.* Amphetamine-induced rotations were different after the lesion (Fig. 6A; group, $F_{2,27} = 5.53$, $P < 0.01$),

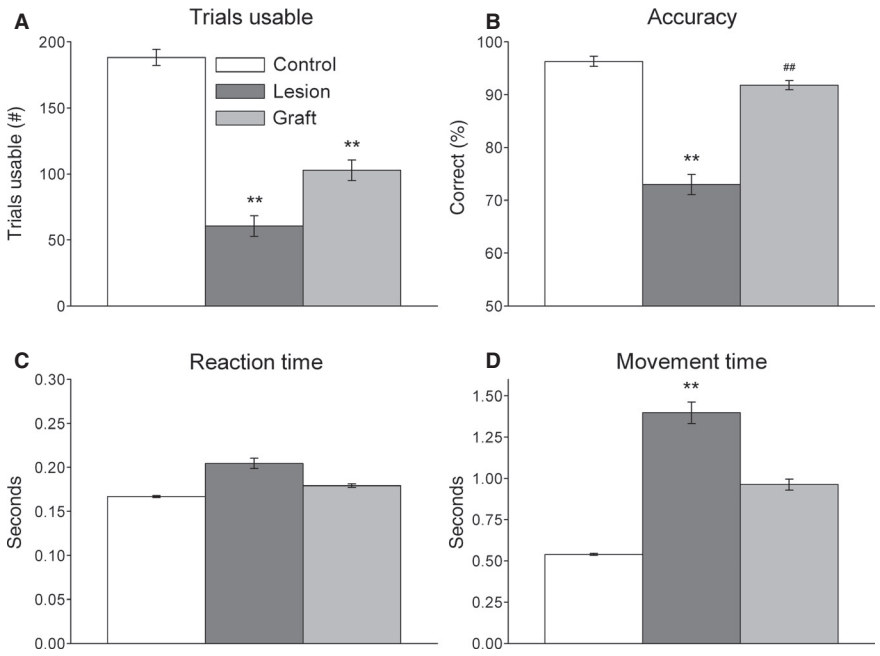


FIG. 4. Main effects of the probe trial. Stimulus duration was increased to 5 s to reduce demand on working memory. All mice produced more usable trials (A) and response accuracy (B). Note that the graft group did not perform significantly different from the control group. As during testing with the standard configuration, the lesion did not significantly affect reaction time performance (C) and, furthermore, grafted mice were not slower in executing the lateralised response than mice of the control group (D). Asterisks denote significant differences from the control group, and hashes denote significant differences from the lesion group, both at $P < 0.01$.

with both lesion groups rotating significantly more towards the side of the lesion than the control group (both, $t > 4.68$, $P < 0.01$). After grafting of one group with dopamine-rich tissue, the difference between the three groups failed to reach statistical significance (group, $F_{2,27} = 2.73$, $P < 0.1$).

*Apomorphine-induced rotations.* Apomorphine-induced rotation assessment was only conducted post-graft. There was a difference between the three groups in the number of rotations per minute (Fig. 6B; group, $F_{2,27} = 6.65$, $P < 0.01$). The lesion-only group rotated significantly more when challenged with apomorphine than untreated mice of the control group ($t_{28} = 5.62$, $P < 0.01$). Mice of the graft group rotated significantly more than mice of the control group ($t_{28} = 3.20$, $P < 0.05$), and although they rotated less than mice of the lesion group, this difference did not reach statistical significance ($t_{28} = 2.42$, NS).

### *Rotarod*

As in previous reports (Heuer *et al.*, 2012b), unilateral lesions to the MFB impaired rotarod performance (Fig. 6C; group, $F_{2,27} = 9.96$, $P < 0.001$), with both lesion groups having a shorter latency to fall than the control group (both, $t > 5.38$, $P < 0.01$). There was a significant interaction between group and week of testing (week × group, $F_{2,27} = 3.66$, $P < 0.05$); after grafting, the graft group performed significantly better than the lesion-only group ($t_{28} > 4.35$, $P < 0.01$).

### *Corridor*

After the lesion, there was a significant difference in the percentage of pellets retrieved (Fig. 6D; group, $F_{2,27} = 8.94$, $P < 0.001$). Mice of the lesion group were significantly more biased to retrieve pellets from the ipsilateral side than mice of the control and graft groups (both, $t_{28} > 3.88$, $P < 0.05$). Mice that received grafts were less impaired than mice of the lesion group, and the difference from the control group did not reach statistical significance ($t_{28} = 2.37$, NS).

## Discussion

In the present study, we aimed to assess the effects of primary fetal tissue grafts rich in dopamine on a series of simple and complex behavioural tasks in the unilateral 6-OHDA mouse lesion model of PD. We found that the lesion-induced deficits were similar to those reported previously, in that the unilateral lesion caused contralateral deficits and impaired task performance on all tests evaluated. Furthermore, VM grafts provided partial amelioration of a subset of the deficits. The effects of the graft were larger when task demands were reduced on the operant task.

### *Lesion-induced deficit*

Injections of the neurotoxin 6-OHDA into the MFB led to a depletion of TH-ir cells in the SN and the VTA on the side of injection, as in previous reports (Grealish *et al.*, 2010b; Francardo *et al.*, 2011; Heuer *et al.*, 2012a,b; Smith *et al.*, 2012a,b). The lesion caused a subsequent depletion of dopamine as determined via the TH marker in the striatum, the nucleus accumbens, the olfactory tubercle, and the prefrontal cortex.

Post-lesion assessment of behavioural performance on the operant task showed a reduction in the number of usable trials, an increase in movement time latencies, more errors in the form of omission of the lateralised response, and a deficit in response accuracy, which was more pronounced when responses had to be made to the side contralateral to the lesion. All deficits on the operant

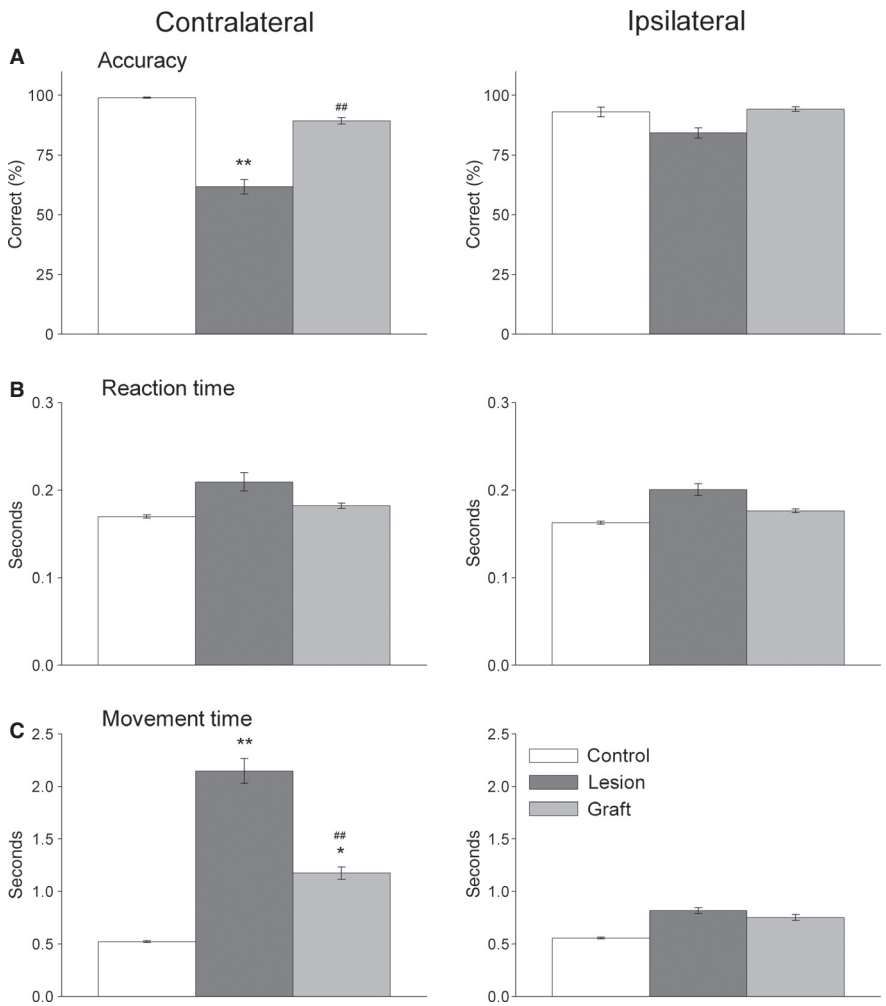


FIG. 5. Main effects of probe trial testing presented per side of response. Whereas there was no difference in response accuracy on the ipsilateral side (A), on the contralateral side grafted mice performed with similar response accuracy to mice of the control group. Reaction time performance was not affected by the lesion on either side of testing (B). Movement time latencies were only increased when mice responded on the side contralateral to the lesion, with both lesion groups showing increased response latencies as compared with the control group, but the graft group responding faster than the lesion-only group (C). The number of symbols denotes significant differences at the $P < 0.05$ (for one symbol) and $P < 0.01$ (for two symbols) levels of significance, respectively. Asterisks denote significant differences from the control group, and hashes denote significant differences from the lesion group.

task were stable on sub-acute and chronic testing. The lesion-induced deficits were similar in all parameters to those in previous studies using the same experimental setup in mice (Heuer *et al.*, 2012a) and rats (Dowd & Dunnett, 2004, 2005a,b). The largest difference between the lesion-induced deficits between the two species is the lack of reaction time deficits in the mouse, although these have been reported in the rat, irrespective of whether testing was conducted in the Skinner box apparatus or the nine-hole box (Dowd & Dunnett, 2004; Heuer & Dunnett, 2012; Heuer *et al.*, 2012a). In the present study, an overall increase (not lateralised) in reaction time latencies was reported in the first week post-lesion and the first week post-graft. Although lesioned mice in this and previous studies showed increased reaction times, the difference was often not significant. The main reason is the large variability in the reaction time data, relative to other parameters. Furthermore, longer delay periods may tease out reaction time difference, but with the cost of having a smaller number of usable trials. The large reduction in the number of usable trials is an additional factor that needs to be considered when interpreting the data of the operant task, as the substantial depletion does certainly cause wide impairment in a number of parameters involving motivation and attention. However, the response profile of the lesion is stable over the weeks of testing, and although lesioned animals make fewer responses, their pattern of responding is highly stable. Previous studies have reported a similar decline in the number of trials started and number of trials usable in rats and mice that were tested on operant tasks (Dowd & Dunnett, 2004; Heuer & Dunnett, 2012; Heuer *et al.*, 2012a).

Previous studies with the nine-hole box, using similar lesions in rats, reported an interesting phenomenon when lesioned animals were first re-introduced into the operant chambers. In the first sessions of post-lesion assessment, the contralateral deficit in response accuracy was not present from the start, but, rather, gradually developed over the first few days (Dowd & Dunnett, 2004, 2007). Even when testing was interrupted and animals were re-introduced to the boxes 12 weeks post-lesion, the deficit, which had been established at sub-acute testing, re-emerged and, as previously, gradually developed over the course of testing (Dowd & Dunnett, 2004, 2007). It was argued that such an 'extinction-like' response profile suggests a reward-signalling function of the nigro-striatal pathway (Schultz *et al.*, 1997; Schultz, 2000; Dowd & Dunnett, 2007). Whereas striatal dopamine is not necessary for the execution of the lateralised

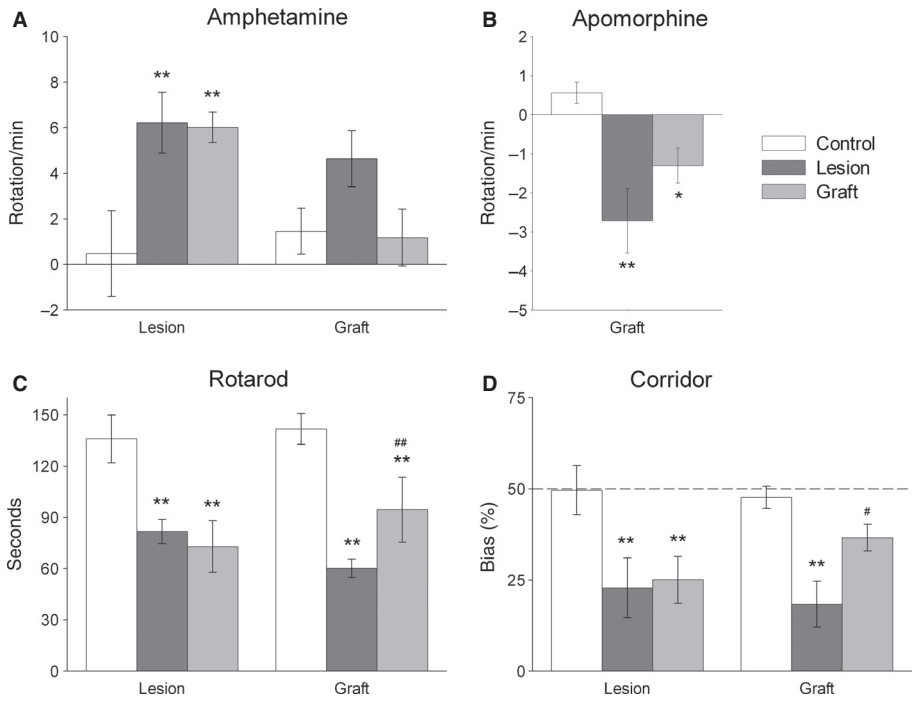


FIG. 6. Tests of simple motor behaviour. Mice of both lesion groups showed turning behaviour towards the side of the lesion (A) when challenged with amphetamine after the lesion. After grafting of one of the lesion groups, the difference in rotational response between any of the three groups was no longer significant. Apomorphine-induced rotations conducted post-graft were higher for both lesion groups than for the control group, but were somewhat reduced for mice that were grafted (B). Whereas both lesion groups were impaired on the rotarod (C) and corridor test (D) after the lesion, mice that were grafted improved in their performance after the grafting. The number of symbols denotes significant differences at the $P < 0.05$ and $P < 0.01$ levels of significance, respectively. Asterisks denote significant differences from the control group, and hashes denote significant differences from the lesion group.

response, but for the maintenance of the stimulus–response association governing the lateralised response, the 'extinction-like' effect seen after unilateral dopamine depletion resembled the extinction curve seen after one side was not rewarded (Dowd & Dunnett, 2007). As in our previous study (Heuer *et al.*, 2012a), we did not find this 'extinction-like' effect in our current study. The reasons for this are not clear, but might be dependent on task parameters and configuration, because, with a different response configuration in the same apparatus, a smaller effect of day was present during acute post-lesion assessment (Heuer *et al.*, 2013), whereas no effect of day was observed with similar lesions when rats were tested in the Skinner box apparatus (Heuer & Dunnett, 2012).

In addition to the operant assessment of the lesion-induced deficits, we investigated the behavioural performance on a number of simple tests of spontaneous motor function. When challenged with the dopaminergic stimulant methamphetamine, mice showed the typical rotational response towards the side of the lesion, confirming the imbalance in striatal dopamine (Ungerstedt & Arbuthnott, 1970; Torres & Dunnett, 2007). The average rotational response (6 r.p.m.) was in between those in previous studies using similar lesions [> 3 r.p.m. (Heuer *et al.*, 2012b) and > 8 r.p.m. (Brundin *et al.*, 1986; Heuer *et al.*, 2012a)], but lower than with lesions to the cell bodies in the SN or the terminals in the striatum (Grealish *et al.*, 2010b; Heuer *et al.*, 2012b). The reason for the reduction in amphetamine-induced rotations after bundle lesions is thought to lie in the substantial degeneration of cells in the VTA and the subsequent depletion of dopamine in the nucleus accumbens. Rats with striatal lesions that received additional, bilateral, lesions to the nucleus accumbens rotated less than those with striatal lesions alone (Kelly & Moore, 1976, 1977), a phenomenon that is well known in the rat (Kirik *et al.*, 1998; Torres & Dunnett, 2007; Grealish *et al.*, 2008). Dopamine in the nucleus accumbens is thought to drive/activate general activity, as lesions to this area resulted in a reduction in the number of trials initiated in rats and mice (Cousins & Salamone, 1996; Dowd & Dunnett, 2004; Heuer *et al.*, 2012a; Heuer & Dunnett, 2013), as well as in reduced locomotor activity. In addition to the drug-induced rotational testing, we investigated the effects of the lesion on the rotarod test, one of the simple motor tests that is most frequently used in mice, and on the corridor test, which has recently been translated and characterised for unilateral mouse lesion models of PD (Dowd *et al.*, 2005; Grealish *et al.*, 2010b; Heuer *et al.*, 2012b). Both tests have previously been shown to be sensitive for detecting clear behavioural deficits in mice with unilateral 6-OHDA MFB lesions (Heuer *et al.*, 2012b). Indeed, both tests reveal stable deficits, with shorter latencies to stay on the rotating rod and a profound ipsilateral bias on the corridor test.

### *Graft-induced recovery*

Some of the profound deficits that were induced by the lesions could be ameliorated by grafts of dopamine-rich tissue harvested from the developing VM. The grafts had similar numbers of surviving TH-ir cells as in a previous study in mice (Smith *et al.*, 2012b), whereas other studies have not quantified surviving cell numbers of intra-striatal grafted cells derived from primary mouse fetal tissue (Brundin *et al.*, 1986; Low *et al.*, 1987; Shimizu *et al.*, 1988). Recent reports of good graft survival and re-innervation when grafts were aimed at the SN are difficult to compare with the present paradigm, as here the cells were not grafted into their original environment (Thompson *et al.*, 2009). The final cell number (1649 ± 342 TH-ir cells) was higher than in a recent report (800 TH-ir cells), where the graft was effective in compensating for the amphetamine-

induced rotational behaviour and graft induced dyskinesias in response to an amphetamine challenge (Smith *et al.*, 2012b).

When discussing the effects of grafts, three factors are important: (i) the model used (ii) the placement of the graft; and (iii) the donor tissue. Here, we used a bundle lesion model which, as discussed above, depletes dopamine not only from the striatum, but also, among other structures, from the nucleus accumbens, the olfactory tubercle, and the prefrontal cortex. These structures are important for optimal performance on the task, as mice use their olfactory sense to explore the world, the nucleus accumbens has classically been implicated in motivation and reward (Fink & Smith, 1980; Cousins & Salamone, 1994, 1996; Salamone *et al.*, 1994; Cousins *et al.*, 1996, 1999; Nicola, 2007), and mice use the prefrontal cortex in planning and decision-making (Joel & Weiner, 1994; Joel *et al.*, 1997; Birrell & Brown, 2000; Robbins, 2002; Chudasama *et al.*, 2003; Dunnett *et al.*, 2005). Restoring dopamine levels via cell grafting into the striatum will not alleviate all deficits. Furthermore, in the present paradigm, cells were not grafted into their original environment, but rather ectopically. The current model provided re-innervation of TH-ir fibres and re-introduction of cells that release dopamine into the depleted areas, but grafting with these parameters does not allow for a full reconstruction of the original circuitry that is lost as a result of the lesion. Recent reports have shown that the A9 type of dopaminergic cells constitutes the important component of a graft for behavioural recovery (Thompson *et al.*, 2005, 2009; Grealish *et al.*, 2010a). In the current model, we grafted cells from the whole developing VM, and therefore a mixture of cells, not all of which will even be dopaminergic.

Despite this, primary fetal tissue grafts were able to ameliorate the lesion-induced deficit on multiple parameters in the simple and more complex tests of motor function that were employed in the present study. On the operant task, mice that received grafts improved in their contralateral response accuracy and showed marginally faster movement times than their lesioned counterparts. Furthermore, they also made fewer omission errors than mice of the lesion group. When the task demand was reduced by increasing the stimulus duration, response accuracy was not significantly different from that in controls. This effect of the graft was therefore stable and different from the effect of lesion only for at least 15 consecutive days of testing ($2 \times 5$ standard and $1 \times 5$ probe configuration). In a previous investigation of primary fetal tissue in the rat, using a similar task, the effect of the graft on response accuracy was different from lesioned rats for only 10 days. After longer testing, lesioned rats improved so much that the difference from the grafted group was no longer significant (both groups at 50% contralateral accuracy), whereas the graft led to sustained improvement on other parameters (trials attempted, premature withdrawals, and movement time) (Dowd & Dunnett, 2004). Furthermore, in the present study, contralateral response accuracy was increased to > 70% at the second week under the standard configuration and to > 89% under the probe configuration. One of the major advantages of operant testing is that task demand and difficulty can easily be adapted to tease out differences between respective groups of animals and interventions.

The significant difference in movement time has to be taken carefully, as both lesion groups already differed significantly on this parameter during post-lesion assessment. This was because of the removal of mice owing to incomplete lesions and/or grafts. Grafted mice did rotate less than lesioned mice on both the amphetamine-induced and the apomorphine-induced rotation tests, although the difference failed to reach statistical significance. Moreover, the failure to detect significant graft effects on drug-induced rotation, a test that is particularly sensitive to graft-mediated recovery in rats, suggests species differences that may reflect practical/physical difficulties in the automated monitoring of rotation in mice, rather than fundamental differences in the underlying pathways of motor control. Rotation analysis in mice is more variable than in rats, and different methods are used. Some studies used video-tracking in an open field (Francardo *et al.*, 2011), or video-recording and *post hoc* scoring (Smith & Heuer, 2011; Smith *et al.*, 2012a), whereas others used automated rotometer bowls (Grealish *et al.*, 2010b; Heuer *et al.*, 2012a). Furthermore, other factors that are not uniformly applied are the method of restriction (harness vs. elastic band), the shape of the environment (glass cylinder vs. bowls), the size of the mouse, and the volume and concentration of injection (Smith & Heuer, 2011). Mice that are restrained too much might not move or freeze, whereas those that are not restrained enough might escape from the harness/elastic band. Many of these problems are not present in the rat, and, if not obtained in a free-moving animal, rotational data might be under-estimated.

In addition to the drug-induced tests, we analysed the effects of the graft on the corridor test and the rotarod test. Grafted mice improved in their performance as compared with lesioned mice and their own post-lesion scores. The corridor test has been shown to be sensitive for detecting dopamine depletion and graft-induced recovery in the unilateral rat model (Dowd *et al.*, 2005; Torres *et al.*, 2008). After a recent characterisation of the corridor task in SN-lesioned mice (Grealish *et al.*, 2010b) and subsequent assessment in MFB-lesioned mice (Heuer *et al.*, 2012b), we report here that, as in the rat, the ipsilateral deficit can be reduced by the graft. The rotarod is one of the standard tests used to assess general motor function, strength and balance in mice, and was shown to be sensitive to the effects of dopamine cell replacement in rat models of PD (Rozas & Labandeira Garcia, 1997). Here, we report similar results, in that grafted mice stayed longer on the rotating drum than their lesioned counterparts.

## Conclusion

In the present study, we have presented a characterisation of lesion-induced deficits on a series of simple and more complex tests of motor function. Lesions produced impairments on all tasks, which were largely lateralised, revealing an impairment in the ipsilateral striatum. Grafts of dopamine-rich tissue were able to ameliorate some, but not all, of the deficits; most notable were the improvements in contralateral response accuracy and rotarod and corridor performance. Both the operant task and the rotarod and corridor tests can be used as drug-free alternatives to assess the effects of cell replacement therapies. Whether novel cell sources can provide improvement of function on more complex behavioural tasks, such as the one presented here, will have to be investigated in the future.

## Conflict of interest

The authors declare no conflict of interest.

## Acknowledgements

We gratefully acknowledge funding from the European Union's Seventh Framework Program NeuroStemCell under grant agreement No. 222943, and support from the Medical Research Council.

## Abbreviations

DMEM/F12, Dulbecco's Modified Eagle's Medium; E, embryonic day; MFB, medial forebrain bundle; NS, not significant; PD, Parkinson's disease; SN, substantia nigra; TH, tyrosine hydroxylase; TH-ir, tyrosine hydroxylase-

immunoreactive; VM, ventral mesencephalon; VTA, ventral tegmental area; 6-OHDA, 6-hydroxydopamine.

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# Chapter 4 General discussion

## 4.1 Summary

The main goal of the series of experiments presented in this thesis was to characterise the effects of unilateral 6-OHDA lesions in rodent models of PD on complex behavioural tasks for cell replacement therapies. I have shown that it is feasible to use the classical choice reaction time task in the Skinner box apparatus, rather than the 9-Hole box (Chapter 3.1) and that the deficit induced by 6-OHDA lesions causes an impairment in directing responses into far contralateral space, regardless of the exact spatial position of the two response options (Chapter 3.2 and 3.3). The lateralised choice reaction time task, and novel versions thereof, are suitable in combination with the bundle lesion model to reveal lasting deficits that are not prone to spontaneous recovery in the rat. Engraftment of DA rich fetal tissue was able to ameliorate some, but not all of the deficits induced by the lesion. In a second strand of experiments three different unilateral mouse lesion models were characterised using a bank of simple behavioural tests (Chapter 3.4). Two of the models (SN and MFB) produced larger lesions than the terminal lesion model and were subsequently used for a first translation of the lateralised choice reaction time task to a mouse model (Chapter 3.5). Again, the bundle lesion model produced a lasting impairment. Subsequently it was shown that engraftment of DA rich fetal tissue was able to ameliorate some of the lesion-induced deficits in the mouse as well (Chapter 3.6). Further improvements in grafting technique, cell survival, and re-innervation might lead to improved recovery of function. We have shown that complex behavioural testing is feasible and necessary for the assessment of cell replacement therapies. In the following discussion the main findings of the presented experiments will be discussed.

# General discussion

This general discussion will be split into two parts. In the first few pages, I summarise the research presented in this thesis. In the latter part, I discuss a few thoughts on cell replacement therapy, methods of grafting and the choice of behavioural models.

## The thesis findings

More work has been conducted on the rat model of dopamine depletion, compared to the mouse, and typically, tests of simple motor function, not higher cognitive function, are employed to investigate the effects of cell replacement therapies. With new transplantation trials quickly approaching and the development of novel cell sources being a primary research focus, it is only a matter of time before stem cell-derived sources will become available to replace the current 'gold standard' of primary fetal tissue.

Whereas the expression of specific biomarkers is certainly important in determining optimal cell lines for therapy, it is also important to index simple motor behaviours, such as overcompensation of the rotational response to amphetamine, as a proof of a surviving and connecting graft. Moreover, investigations of the impact of cell replacement therapies on non-motor and higher cognitive function is critical. Thus, it is imperative to characterise fully the lesion models they are tested on and, furthermore, to investigate the deficits that these cell replacement therapies can/cannot ameliorate. The ultimate question will then be of how these novel cell sources compare to the 'gold standard' primary fetal tissue.

The experiments presented in this thesis were aimed at the characterisation of lesion-induced deficits on more complex behavioural tasks of motor and cognitive function for the purpose of the assessment of cell replacement therapies. In the unilateral 6-OHDA lesion model of PD so far operant assessment of primary fetal rat tissue grafts has utilised choice reaction time tasks. Therefore I aimed to utilise versions of the lateralised choice reaction time task to investigate the lesion-induced deficits and the potential of cell replacement therapies to alleviate the deficit. As interest in mouse models has increased over recent years, and these have not been sufficiently characterised, I aimed to assess basic and more complex lesion-induced deficits. After comparing three types of lesions along the nigro-striatal pathway on a histological and behavioural level, I investigated two of the models that produced the largest depletion on the mouse version of the lateralised choice reaction time task.

Once the basic task parameters and the lesion-induced deficit were assessed on the task I conducted a primary grafting experiment to show that primary fetal tissue grafts are able to ameliorate the lesion induced deficit in a similar way to the rat.

The first manuscript '*unilateral 6-OHDA lesions induce lateralised deficits in a 'Skinner box' operant choice reaction time task in rats*' translated choice reaction time task from the most commonly used rat lesion model of PD from the 9-hole box to the Skinner box. Whilst even in behavioural laboratories the 9-hole is a rather esoteric piece of equipment, the retractable lever Skinner box is more widely available. The lateralised choice reaction time task has been used in the Skinner box previously utilising intra-striatal excitotoxin infusion as a model for HD, but DA depletion via unilateral 6-OHDA infusion to the MFB has, to our knowledge, never been attempted.

Whereas an earlier study had shown that lesioned animals do display spontaneous recovery on the Skinner box version of the lateralised choice reaction time task (Heuer & Dunnett, unpublished observation), in the present manuscript surgical and task parameters have been adapted to produce lesions that resulted in a stable and chronic deficit in dopamine-depleted rats. The failure of previous experiments was mainly due to incomplete lesions and differences in task design/parameters. Firstly, during an initial experiment using exactly the protocol described by Döbrössy and Dunnett (1997), rats that were lesioned via unilateral 6-OHDA infusion into the MFB did recover on their accuracy within three weeks of post-lesion testing, despite all lesioned rats displaying initial impairments during the first week of testing after the lesion. Although the data provided some insights into the neuronal function of DA, as the main aim of the present work was to assess the viability of complex behavioural tests for assessing cell replacement therapy, the recovery seen made this impossible. In our laboratory and other groups the success of the unilateral 6-OHDA MFB lesion using this protocol dropped substantially and the recovery seen above could be ascribed to incomplete lesion (Torres *et al.*, 2011). With the readjustment of the lesion co-ordinates, keeping all other parameters the same (concentration, flow-rate, volume, cannula size), we achieved a > 90% lesion success rate in the subsequent experiments presented in this thesis. The second parameter that was changed in respect to the previously mentioned study was the duration of the lateralised stimulus light. Whereas in the previous studies the light was signalling the correct response until a response had been made (5 sec) (Dobrossy & Dunnett, 1997; 1998), in the current experiment the stimulus duration was limited to 1 sec. This reduction is thought to increase demands on working memory as the rat has to

keep the location of the stimulus in mind, rather than being able to rely on the stimulus light being used as a beacon. We have shown that under these parameters, the lateralised choice reaction time task conducted in the Skinner box apparatus is a powerful tool for the analysis of lesion-induced deficits. The results obtained are comparable to the deficits reported when experiments had been conducted in the 9-hole box.

A previous comparison between the two boxes had shown that the task can be used in principle, but was less sensitive in measuring deficits in reaction time, when assessing the effects of intra-striatal quinolinic acid lesions as a model of HD (Brasted *et al.*, 1998). In the present experiment (Chapter 3.1) we reported a deficit in all parameters, including reaction time performance (Heuer & Dunnett, 2012) giving further support to the sensitivity of the Skinner box apparatus with the current configuration. All deficits were stable sub-acute and long-term, making the task/apparatus useful as a tool for the assessment of cell replacement therapies.

In the second manuscript *'Dopamine-rich grafts alleviate deficits in contralateral response space induced by extensive dopamine depletion in rats*' we characterised further the lesion-induced deficit on a version of the lateralised choice reaction time task. Previous studies have shown the effect of contralateral impairments in response accuracy in the 9-hole box that we reported in the Skinner box in chapter 3.1 (Carli *et al.*, 1985; Carli *et al.*, 1989; Dowd & Dunnett, 2004; 2005a; b). Furthermore, when rats were presented with a near and a far response location, rather than responses being presented to either side of the animals' head, intra-striatal administration of 6-OHDA led to a neglect of the far contralateral response whilst responding to the near contralateral side was intact (Brown & Robbins, 1989b). Lesions made ipsilateral to the side of testing did not have any statistical significant effect on main task parameters intact (Brown & Robbins, 1989b). This version of the task provided a more in-depth analysis of the lesion-induced deficit as it measures an additional dimension to the standard bilateral two choice condition. In order to employ this task for the assessment of cell replacement therapies, we needed to characterise it for the bundle lesion model and to show that the lesion induced deficit is stable over long-term testing, since terminal lesions were employed in the Brown and Robbins (1989) study. Terminal lesions are in general less complete than infusions to the MFB as they largely spare most cells in the VTA as well as some nigral cells, whereas MFB lesions will produce a near complete loss of nigral neuons as well as substantial VTA loss when targeted correctly. Terminal lesioned rats have been

shown to be prone to spontaneous recovery on the standard configuration of the choice reaction time task, as discussed above (Dowd & Dunnett, 2005a). In the submitted manuscript we showed that (i) the lesion produces a deficit that is similar to that reported after terminal lesions (Brown & Robbins, 1989b), although we reported an impairment on the ipsilateral side of the lesion as well (Heuer *et al.*, 2013a); (ii) the lesion-induced deficit was stable during both acute post-lesion and long-term assessment. Even after 50 consecutive days of testing there was no sign of recovery on any of the parameters assessed. As discussed above, the lack of spontaneous recovery is imperative to assess the effects of cell replacement therapies so that the beneficial effects of the graft are not obscured. Although the effects of the graft do encompass more parameters than response accuracy, this parameter is one of the most interesting as it represents recovery of a deficit that is not primarily motor or sensory in nature. In one study published on the effects of nigral grafts in standard configuration of the lateralised choice reaction time task, the effect of response accuracy seems to be different from lesioned rats only for 10 days of long term testing (see Dowd and Dunnett, 2004). However, rats of the lesion group did exhibit some recovery over the last two days of testing, so that the difference to the graft group was not significant, despite the near-complete lesion (Dowd & Dunnett, 2004). We have shown here that the current adaptation of the lateralised choice reaction time task, including a near and far response location, is sufficiently sensitive to reveal a stable effect of the lesion as well as to separate lesioned and grafted rats on multiple parameters. Rats that were grafted with DA-rich fetal cells attempted more trials, produced a higher number of correct responses to the previously neglected, contralateral far response location and displayed movement time latencies not significantly different from the control group. The grafts also had an effect on ipsilateral accuracy. The contralateral near hole bias displayed by lesioned rats was not affected by variations of the stimulus duration or by shifting the response location. Although grafted animals do not recover to the level of controls, the improvement by the grafts is remarkable, as lesioned rats do produce very few responses to the contralateral far hole. This is, to our knowledge, the first study assessing the sensitivity of this type of choice reaction time task in MFB lesioned rats and grafted rats and will therefore serve as a guide for future studies.

In the third manuscript '*Characterisation of spatial neglect induced by unilateral 6-OHDA lesions on a choice reaction time task in rats*' we investigated an interesting phenomenon that emerged from the previous experiments. Although rats were tested over more than 50 consecutive days, lesioned rats did not recover on their far

contralateral response accuracy. Although we did not expect full recovery, some improvements with repeated testing were anticipated. Furthermore, lesioned rats in experiment 1 not only displayed a contralateral accuracy deficit, but when they responded towards the contralateral lever, they did so with a full body turn, rather than a direct (and shorter) movement. These two observations led us to speculate that lesioned rats adapt their response strategy as a result of the dopamine depletion. If rats in experiment 1 biased all their responses towards the contralateral near hole they would still get rewarded on 50% of the trials. In experiment number 3 we implemented an error correction procedure that would present the same trial to the rat again, when an incorrect response had been made. Consequently, if the rat biases all responses towards the contralateral near hole it will receive no further rewards after the first correct response. The results suggest that rats do not display a shift in response strategy but rather, as previously suggested, a failure to direct responses accurately in far contralateral space (Brown & Robbins, 1989b; Brasted *et al.*, 1997; Heuer & Dunnett, 2013), i.e. all lesioned rats continued to display a near-hole bias when tested on the contralateral configuration. We manipulated the configuration further in a series of probe trials which showed that the response deficit was irrespective of the absolute position in space, i.e. shifting of the absolute response location shifted the deficit and the lesioned rats directed almost all their contralateral responses towards the more proximal response location. Furthermore there seems to be some overlap between the two striata as no contralateral deficit could be detected when the response location was immediately adjacent to the centre hole in a simple reaction time setting, but only when the response location was further away. However, even when the contralateral response location was most distal, the deficit was smaller in a simple reaction time setting when compared to when making a less distal choice response. This study gave further insight into the lesion-induced deficit and the power of the choice reaction time task (and its variations) to investigate DA depletion models in rats.

Thus, the first three experimental chapters presented in this thesis have provided further insight into the unilateral 6-OHDA near complete lesion model of PD in the rat. The deficits caused by the extensive, near-complete DA depletion (>95%) on the task are manifold and encompass motivational, attentional, motor and cognitive processes due to the DA depletion in multiple areas of the brain.

The second strand of research in my thesis was concerned with the development of the 6-OHDA lesion model of PD in the mouse. Whereas some early studies did use

the mouse to model PD (Von Voigtlander & Moore, 1973; Mandel & Randall, 1985; 1990; Mandel *et al.*, 1992), the majority of studies in the last 40 years have focused almost entirely on utilising rat. The major reasons are that rats are relatively easy to handle and house and they are more robust to insults than mice. There is also a long and rich psychological literature developing a very wide range of tests and tools for behavioural analysis in the rat. As a tool for transplantation and to model the DA loss of PD the rat lesion model has been the gold standard since the 1960s. On the other hand, mice have the advantage that genetic manipulations are more readily inducible, e.g. GFP mice can be used as donor tissue to trace grafted fibre outgrowth, and mice with specific gene mutations – whether using transgenic, knockin, knockout or conditional gene expression technologies – can be combined with lesions to mimic different aspects of the disease. In recent years a number of studies have revitalised the mouse lesion model of PD (Lundblad *et al.*, 2004; Lundblad *et al.*, 2005; Grealish *et al.*, 2010b). With the introduction of intensive post-operative caring regime mortality rates have been at acceptable levels and mouse testing has become more feasible (Francardo *et al.*, 2011; Smith & Heuer, 2011; Heuer *et al.*, 2012; Smith *et al.*, 2012a). Whereas previous work has characterised one or two aspects of the unilateral 6-OHDA lesion model, no comparison has been done with injections to any of the three main sites of the nigro-striatal pathway.

In the fourth manuscript, entitled '*Unilateral nigrostriatal 6-hydroxydopamine lesions in mice I: Motor impairments identify extent of dopamine depletion at three different lesion sites*', I have therefore compared the three most widely used nigro-striatal lesion models on a series of simple-motor tests. We showed that mice with a loss of >80% of TH-ir cells in the SN displayed impairments on the corridor test and rotarod apparatus, in addition to amphetamine- and apomorphine-induced rotation tests. The results obtained are in line with work that was published shortly after the current experiment had been completed (Grealish *et al.*, 2010b). Grealish et al (2010) showed that a combination of tests is useful to assess the size of the lesion. Both in studies of drug-induced rotation as well as using the cylinder test they were able to detect lesion-induced deficits. One recent report by Francardo et al. (2012) does provide a comparison of the three lesion models in the development of l-DOPA induced dyskinesias, but behavioural assessment was limited to a few simple behavioural tests (Francardo *et al.*, 2011). Thus, whereas the former paper (Grealish *et al.*, 2010b) only employed one type of lesion (SN) and the latter provides a comparison of all three lesion models (Francardo *et al.*, 2011), we here presented the behavioural performance of all three lesion types on a wider array of behavioural

tests. The results presented can help to guide future studies to choose the best lesion mode, depending on investigation, the degree of DA depletion wanted and which behavioural test to use to select successfully lesioned mice. The present manuscript has shown that mouse models of PD are feasible to use as long as some aspects are considered, which will be discussed below.

Firstly, apart from the obvious differences, mice are not just small rats. Thus, mice are easier to startle; are more anxious; are less robust to insult; typically exhibit a higher mortality after lesion; and they can utilise different strategies on behavioural tests such as the Morris water maze (Frick *et al.*, 2000). When conducting behavioural testing in mice, greater attention is required to testing procedures to achieve optimal performance. Furthermore, whereas unilateral near-complete lesion via 6-OHDA infusion into the MFB in the rat virtually never causes mortality, the same procedure in the mouse has been reported to cause mortality in some studies greater than 80% (Lundblad *et al.*, 2004; Grealish *et al.*, 2010b). In the context of animal welfare, ethics and regulation, this is not acceptable as a routine strategy. Moreover, whereas rats can be considered more exploratory and display diverse foraging and predator-like behaviour, mice certainly exhibit more similarities to a prey species, and the resulting differences in species-specific behaviour have to be taken into account in the design of appropriate assessments. As shown in manuscript number 4, the cylinder test, which is frequently used in the rat, was less sensitive to detect lesion-induced deficits, whereas the corridor test, which is not as frequently used, does distinguish well lesioned from unlesioned mice.

In the fifth manuscript, entitled '*Comparison of 6-hydroxydopamine lesions of the substantia nigra and the medial forebrain bundle on a lateralised choice reaction time task in* mice', we aimed to establish and characterise the classical choice reaction time task from the rat to the mouse (Carli *et al.*, 1985; Heuer *et al.*, 2013b; Heuer *et al.*, 2013c). We showed that mice, similar to rats, were impaired on a range of parameters of performance, including trials usable, response accuracy and movement time. As shown previously, bundle lesions produced the more complete DA degeneration and subsequent behavioural impairment, compared to the other two infusion sites (Dowd & Dunnett, 2005a; b; Heuer *et al.*, 2013b). Although the overall impairment was similar between the two species, there were some differences as well. Firstly, the deficit in the rat was more lateralised, with no significant impairment on ipsilateral response accuracy, whereas in the mouse we reported a bilateral impairment that was more pronounced on the side contralateral to the lesion on this

task parameter (Dowd & Dunnett, 2005b). Secondly, reaction time was significantly increased in the rat whereas in the mice the difference between the bundle lesion and the control group failed to reach statistical significance in the post hoc analysis. For other task parameters, such as movement time, mice performed with a similar pattern as reported for the rat (Dowd & Dunnett, 2005b). Interesting, whereas terminal lesioned rats with a 70% reduction in the number of TH-ir cells on the lesioned side were not impaired in movement time and only marginally impaired during acute post-lesion testing on task accuracy (Dowd & Dunnett, 2005a), SN lesioned mice were impaired in the present studies on these task parameters. The main difference can be attributed to the larger sparing of remaining TH-ir cells in the ipsilateral SN in the rat terminal lesion group, compared to the mouse SN lesioned group (<25% TH-ir cells left). Similar to the rat, a deficit that at least encompasses a 80% reduction will produce stable lesions.

Because of the successful adaptation of the choice reaction time task, in the final study presented in the manuscript entitled '*Behavioural recovery on simple and complex tasks by means of cell replacement therapy in unilateral 6-OHDA lesioned mice*' I tested whether DA rich fetal grafts can ameliorate lesion-induced deficits in mouse PD models similar to the recovery seen in rats. Only few studies have been published using primary fetal VM as donor tissue in mice as hosts, and almost all focused on drug-induced rotations, rather than drug-free and/or more complex tests of motor behaviour. Despite this significant improvement, not all parameters were recovered and even though response accuracy increased, the grafted mice still performed at a level below that of the control animals. The failure of full recovery by primary fetal VM tissue grafted into the denervated striatum will be discussed in further detail below. Despite the fact that 'full' recovery was not achieved (similar to Experiment 3.2), the effects of the graft were remarkable. Although both, the lesion and the graft groups, had similar levels of depletion, with a near complete absence of TH-ir cells in the SN, the latter group did improve the lesion-induced deficits on a number of parameters. These improvements could be detected on simple and complex behavioural tests. Specifically, DA-rich grafts led to amelioration on contralateral response accuracy on the choice reaction time task as well as improved motor function on the rotarod test and the sensori-motor neglect on the corridor test. Although grafted animals did not display a rotational bias, unlike lesioned mice, at the post-graft time-point, this difference failed to reach statistical significance. This is most likely due to the large variation seen in these data. Whereas all other parameters measured are analysed of averages of multiple sessions, thereby

reducing variability, rotation tests were conducted only the once. In our hands, the use of automated rotometer bowls can lead to an underestimation of the actual rotation score. Although the use of this automated system makes testing easy and quick, it is hampered by the restriction of the animal to the elastic band. Whereas a similar procedure in the rat does not seem to affect (or bother) the animal, mice do try to escape from the restriction. Therefore the restriction has to be balanced, between too loose so the mouse does not escape and too tight so the mouse is not restricted in its movement. Videotaping or another restraining method might circumvent this problem. From our experience and even the data provided in Chapter 3.4 and 3.6 mice do rotate, similar to rats, in response to DAergic drugs such as amphetamine and apomorphine. Correlations between rotation scores and TH-ir cell loss in the ipsilateral SN were not possible due to the small variation in the remaining cells as all animals displayed a large loss of the cells. However, on the other tests, grafted mice certainly benefitted from the graft for the three weeks of operant testing and the week of simple motor behavioural testing. Especially during the probe trial on the operant task grafted mice were not significantly different to mice of the control group in their movement times and response accuracy, despite lesioned mice displaying an impairment on these parameters even under the condition where task demand was lower than during the standard configuration. Furthermore grafted mice had fewer procedural errors in terms of omissions at both post-graft time points, compared to mice of the lesion group. The data presented will provide a baseline of simple and complex behavioural test that can be alleviated by a DA-rich graft. Alternative cell sources that are currently developed will have to hold up the performance of the 'gold standard' that has been presented in experimental chapter 3.6.

### Mortality in the unilateral 6-OHDA mouse lesion model of PD

Whereas the unilateral 6-OHDA lesion is relatively easy to achieve in the rat, similar lesions in mice have been hampered for several reasons, but mainly due to the high mortality rate that is associated with near complete lesion when the toxin-infusion is aimed at the MFB (Lundblad *et al.*, 2004; Grealish *et al.*, 2010b). For the purpose of the work presented in this thesis a stable lesion is needed as the aim of the work is to characterise tests that are sensitive to detect lesion-induced deficits and subsequent-induced recovery. Only a lesion that is stable over long term testing will provide the baseline against which to measure the intervention. Whereas mortality in unilateral lesioned rats is very rare, similar lesions produce a high level of mortality in mice. With the introduction of an intensive post-operative caring regime mortality rates

could be reduced to an acceptable level (<10%) in our and other studies (Cenci & Lundblad, 2007; Francardo *et al.*, 2011; Smith & Heuer, 2011; Heuer *et al.*, 2012; Smith *et al.*, 2012a; Smith *et al.*, 2012b; Heuer *et al.*, 2013b). After the lesion, mice are prone to dehydration and excessive weight loss can follow, especially in animals with a good lesion. Observing the mice in their home cage clearly shows that they rotate spontaneously and this rotation is much higher than that of seen in rats receiving similar lesions. Mice that attempt to drink and eat from the snout of the water bottle or food-pellet hopper rotate away, rather than standing still to feed. Furthermore unlesioned/less depleted mice do bully weaker animals away from food/water. Three measures have been implemented in the current work which allowed us to use the unilateral 6-OHDA MFB lesion model in an acceptable fashion in mice; (i) mice were montiored every day for a minimum of 14 days post-lesion to detect weight loss early. The researcher then is able to intervene before the critical weight has been reached; (ii) mice are closely monitored in their home cages and bullied mice are promptly separated from the aggressor; (iii) dehydration and weight loss is tackled by daily injections of 0.5ml of glucose-saline solution s.c. three times daily and providing the animals with palatable wet mash in multiple containers in their home cages 2-3 times daily. This procedure is laborious, especially if large cohorts are tested, but after 10-14 days of intensive post-operative care mortality rates can be reduced to acceptable levels. However, once these measures are taken, the unilateral near-complete mouse lesion provides a powerful model to investigate grafting of mouse-derived DAergic stem cells without the need for immunosuppression, and can be used as readily in genetically-modified as in wildtype animals. A great deal of data has now been accumulated by other groups as well as ours to select the appropriate lesion model and to identify behavioural tests that are sensitive to detect different degrees of DAergic cell loss in the SN (Lundblad *et al.*, 2004; Iancu *et al.*, 2005; Lundblad *et al.*, 2005; Cenci & Lundblad, 2007; Grealish *et al.*, 2010b; Smith & Heuer, 2011; Heuer *et al.*, 2012; Smith *et al.*, 2012a; Smith *et al.*, 2012b; Heuer *et al.*, 2013b).

### Graft placement

In the present model we grafted tissue into the denervated striatum ectopically rather than homotopically into the SN from where the host nigral cells are lost. Although this ectopic graft placement does lead to recovery on numerous parameters and tasks, as stated above, it only allows for a limited recovery.

The lesion used in the present model was chosen mainly because of the stability of the lesion and the large depletion of DA that is produced by the current procedure. Lesion-induced deficits are stable over long-term testing and therefore behavioural recovery by the transplants can confidently be ascribed to the intervention (the graft) rather than spontaneous recovery. The downside of the present model is that the toxin infusion into the MFB leads to a destruction of cells not only in the ipsilateral SN but also to a large degeneration of cells located in the adjacent VTA. The result is a depletion of DA from not only the striatum but also from the nucleus accumbens, the olfactory tubercle, the thalamus and the prefrontal cortex (Kirik *et al.*, 1998; Dowd & Dunnett, 2004; Grealish *et al.*, 2008; Walsh *et al.*, 2011). Grafting into the striatum leaves remaining structures without DA and therefore it is unlikely that the graft has a (direct) effect on these. Although grafted fetal DAergic cells do re-innervate the immediate surrounding host area, usually seen via the TH-ir 'halo' that surrounds the graft core, the re-innervation is less extensive than is seen in the intact SN, where one cell in the SN sends out projections to the striatum where the axon terminals undergo massive ramification and form up to 250,000 connections (Yurek & Sladek, 1990). Primary fetal tissue transplants do form connections with the host tissue (Doucet *et al.*, 1989; Doucet *et al.*, 1990). However as allograft re-innervation is confined to a relatively small location, Winkler et al. (1999) and others have grafted multiple smaller deposits ('microtranplants') throughout the striatum in order to achieve greater re-innervation (Nikkhah *et al.*, 1993; Nikkhah *et al.*, 1994b; Winkler *et al.*, 1999; Nikkhah *et al.*, 2009). Interestingly, intra-nigral transplants alone had some effect on postural-balance and skilled forelimb use but the benefits offered by the intra-SN graft have never matched the efficacy of grafts placed into the striatum (Winkler *et al.*, 1999). Intra-SN grafts of GABAergic cells on the other hand did improve behaviour on paw reaching, and this effect was larger than by intra-striatal placed grafts of DAergic tissue. By combining the two approaches, GABAergic grafts into the SN and DAergic grafts into the striatum led to the best – although still incomplete – behavioural recovery (Winkler *et al.*, 1999). Striatal fibre density was actually increased to 50% of normal in the caudal striatum, 60% in the nucleus accumbens and central striatum and 75% in the rostral striatum. The authors state that the re-innervation of TH-ir was between 45% and 80% to that of controls. Despite this large re-innervation by microtransplantation and the additional effects of intra-SN grafts of GABAergic cells, behavioural recovery was only complete on the drug-induced rotation test, with the tests that require a more complex behavioural response not recovering to normal, despite large improvements when compared to rats of the lesion only group (Winkler *et al.*, 1999). The authors concluded that a

minimal re-innervation of 30% of control is necessary to detect functional effects by the graft, but that any additional innervation does not lead to greater recovery (Winkler *et al.*, 1999). By using the micro-transplantation approach, rather than grafting the cells in one large deposit, re-innervation is greater by a factor 2.5 and survival by a factor 2.8, compared to the latter approach (Nikkhah *et al.*, 1994b). Other double-grafting paradigms, by placing cells simultaneously into the SN and the striatum, have been shown to produce greater innervation and faster behavioural recovery, although this has only been assessed using drug-induced rotations (Baker *et al.*, 2000; Mendez *et al.*, 2000). When assessing lesion-induced deficits and graft-induced recovery, the majority of studies utilize the drug-induced rotation model. It is important to note that, although the rotational response to amphetamine gives a good indication of the size of the lesion/nigro-striatal degeneration, graft size does not influence the rotational response once a certain number of surviving cells is reached. This ceiling effect has been estimated to be about 100-200 DAergic cells necessary to reduce the rotational response to amphetamine by 50%. Larger grafts (300-500 cells) did not produce more alleviation (Brundin *et al.*, 1988).

Interestingly, even though there is a large re-innervation throughout the striatum (up to 80% of control in some animals), behavioural deficits were still detectable. This further shows that replacing the DA lost is by itself insufficient to induce full behavioural recovery. Indeed, a 20% depletion would be difficult to detect and most likely be prone to spontaneous recovery (Kirik *et al.*, 1998). In another interesting study, the effects of intrastriatal nigral grafts were compared on the extent of the lesion (Kirik *et al.*, 2001b). Graft survival and outgrowth was lower in rats with partial lesions and graft-induced recovery could actually only be detected in rats that had a depletion level of larger than 70% (Doucet *et al.*, 1990; Kirik *et al.*, 2001b). However, in rats in which the lesion was confined to the striatum greater recovery could be detected (Kirik *et al.*, 2001b). This can be taken as further evidence that the near-complete lesions produce deficits that cannot be recovered by intra-striatal nigral grafts, mainly due to the extra-striatal pathology. Interestingly, these two findings pose opposite challenges/opportunities for translation to the clinic. On the one hand grafts exert their largest effect when grafted into a partial lesion, in particular when DA depletion in the patient is restricted to the striatum. On the other hand, at that early stage, due to the remaining DA-innervation, graft survival and fibre outgrowth is restricted due to the non-permissive environment (Kirik *et al.*, 2001b). In line with the ideas about circuit reconstruction it can be assumed that the tonic DA release by ectopically place grafts is necessary, but not sufficient to induce behavioural recovery

on more complex tests. Similar to the pharmacological action of l-DOPA, tonically released DA does alleviate deficits on simple motor behaviour. Placing the graft homotopically into the SN would theoretically circumvent this problem if the graft would make the appropriate connections and re-innervations.

Homotopically placed grafts into the SN of rats and mice do survive, although there were mixed reports about their axonal outgrowth (Bjorklund *et al.*, 1983b; Dunnett *et al.*, 1983a; Nikkhah *et al.*, 1994a; Nikkhah *et al.*, 1995; Winkler *et al.*, 1999; Winkler *et al.*, 2000; Thompson *et al.*, 2009; Kauhausen *et al.*, 2013). In most reports fibre outgrowth remains in the proximate areas surrounding the graft and does not reach the striatum (Nikkhah *et al.*, 1994b; Dunnett, 1999a; Winkler *et al.*, 1999) It has been suggested that inhibitory factors prevent grafted tissue to grow the distance to the denervated target area (Fawcett, 1997). Interestingly, xenogenic tissue is able to grow further suggesting that the inhibitory effect is tissue specific, for example, transplanted human dopaminergic fibroblasts were able to grow a distance up to 10 mm and innervate the striatum, amygdale, frontal cortex and the olfactory bulb. (Wictorin *et al.*, 1990a; Wictorin *et al.*, 1990b; Wictorin *et al.*, 1992; Barker *et al.*, 1999; Barker *et al.*, 2000). Furthermore, the inhibition seems to be specific for adult host tissue as transplants that were grafted into neonatal rats did show remarkable fibre outgrowth (Nikkhah *et al.*, 1995). This approach led to one of the largest behavioural effects reported to date using DAergic cell replacement in 6-OHDA lesioned rats. Despite the large recovery on rotation tests and skilled forelimb use, as previously, not all deficits were recovered. Bridge-grafts have been used to grow axons from the SN along a bridge of Schwann cells into the striatum where their terminals were re-innervating the striatum. These type of grafting technique allowed for a reduction in amphetamine-induced rotations and increased graft survival compared to rats that received grafts but no ‘bridge’ between them (Brecknell *et al.*, 1996a; Brecknell *et al.*, 1996b). Other studies have shown with similar techniques that bridges between the SN and the striatum produce greater graft survival, in terms of cell numbers, and allow for TH-ir axons to grow along the bridges and reach the striatum. Not each type of tissue is usable for providing viable ‘bridges’; better results have been achieved using sciatic nerve (Aguayo *et al.*, 1984), Schwann cells (Brecknell *et al.*, 1996a), and striatal or olfactory neuron bridges (Dunnett *et al.*, 1989), but not laminin-coated beads or astrocytes (Dunnett *et al.*, 1989). Furthermore, of the four types of cell-bridges used by Dunnett *et al.* (striatal, olfactory, astrocyte, laminin), only rats that received striatal bridges and displayed TH-ir fibre innervation of the striatum displayed a reduction in methamphetamine-

induced rotation scores (Dunnett *et al.*, 1989). Using bridge grafts to provide the homotopicially grafted DAergic cells with a 'scaffold' or 'trajectory' to grow along, has shown to provide greater innervation of the striatum than without, although overall innervation pattern were still low (Aguayo *et al.*, 1984; Brecknell *et al.*, 1996a; Brecknell *et al.*, 1996b; Dunnett, 1999a). However, although graft placement is important for the recovery of certain functions (Dunnett *et al.*, 1981c; Dunnett *et al.*, 1983a; Dunnett *et al.*, 1984; Dunnett *et al.*, 1988a), it is not the only important factor to consider (Barker & Dunnett, 1999). When comparing wGE grafts in the QA lesion model and VM grafts in the 6-OHDA lesion model, behavioural recovery does not only depend on lateral graft placement but also on the type of tissue used. Whereas in the former model recovery on skilled paw use in a reaching test is improved, the graft in the PD model does not show any beneficial effects, despite being placed in the same location within the striatum (Dunnett *et al.*, 1988b; Montoya *et al.*, 1990; Barker & Dunnett, 1999).

The tissue used for primary fetal grafts consists not only of DAergic neurons, but rather of a mixture of different cell types. Recently it has been shown that the nigral A9-type of cells in particular are needed for greater innervation and functional behavioural improvements (Grealish *et al.*, 2010a). With the development of new techniques such as cell sorting, cell labelling, and stem cell technologies (e.g., reporter cell lines) it will be possible to provide a more pure cell suspension, which can help to resolve the theoretical issue and in turn may provide a better functional outcome. Additionally, these new techniques will allow for a better visualisation of the graft and which cell populations are important for what aspect of recovery, e.g., A9 grafts for general motor improvement and astrocytes for better graft survival.

Recent studies in rats and mice have shown that the use of younger donor tissue provides larger grafts, which is mainly due to improved survival and a larger proportion of the A9-type cells (Thompson *et al.*, 2005; Torres *et al.*, 2007; Torres *et al.*, 2008a; Thompson *et al.*, 2009; Bye *et al.*, 2012). Advances in stem cell technologies will eventually provide pure populations of certain cell types, although at present, there are a lack of markers and transcription factors. Although A9 and A10 type of cells can be distinguished on their relative co-expression of GIRK-2 or Calbindin with TH, this distinction is not absolute as there are cells that display considerable overlap of the respective markers (Thompson *et al.*, 2005; Bye *et al.*, 2012). Furthermore developmental factors as LMX1A or FOXA2 are currently used to confirm that cells are developing into a DAergic lineage (Nelander *et al.*, 2009;

Pfisterer *et al.*, 2011a; Kirkeby *et al.*, 2012), but there is as yet no conclusive marker that can be expressed to confirm full developmentof the A9 phenotype. New technologies in the form of induced pluripotent stem cells (iPS) (Takahashi & Yamanaka, 2006), direct induced neurons (iN) (Pfisterer *et al.*, 2011a; Pfisterer *et al.*, 2011b), embryonic stem cells (ES) (Kriks *et al.*, 2011) or cells from expanded primary fetal VMs will help to generate 'pure' A9-type cell populations eventually, although at the moment the race is still open with primary fetal tissue remaining the currently availble 'gold standard'.

### Grafting parameters

Greater re-innervation can mainly be achieved by combining findings from multiple lines of research. Usually laboratories change one parameter at a time to investigate the effects of specific manipulations of cell numbers transplanted or the effect of trophic factors, but when translating the findings to the clinic a combined approach has to be considered first. In the paradigm used by Winkler et al. (1999), combined grafts of intrastriatal transplanted DAergic grafts and intra-SN transplanted GABAergic grafts had additive effects (Winkler *et al.*, 1999).

Larger grafts and more extensive re-innervation can be achieved via several methods. First, it is important to note that the transplanted cell suspension, derived from an entire VM dissection consists of, only 5-11% (about 35,000 cells in the rat) of cells that are actually DAergic (Sauer & Brundin, 1991; Nakao *et al.*, 1995; Schierle *et al.*, 1999). Moreover, the majority of the grafted cells die within the first 6 days after transplantation (Brundin & Bjorklund, 1987). The mechanisms responsible for cell death have been identified as apoptosis, injury, oxidative stress and energy deprivation, to which the DAergic subpopulation is particularly susceptible (Brundin & Bjorklund, 1987; Castilho *et al.*, 2000). Improvements in dissection technique, cell preparation, and transplantation are directly under the control of the investigator. Dissections should be narrow to exclude the non-DA cells away from the midline and relatively rostral to exclude the large caudal population of serotonergic cells; handeling of the preparation should be done with utmost care to prevent damage to the cells; transplantation should occur producing a minimum of trauma to the host brain; the use of the right donor age (Torres *et al.*, 2007; Torres *et al.*, 2008a; Kauhausen *et al.*, 2013), preparation of the host environment by trophic factors (Sinclair *et al.*, 1996; Ahn *et al.*, 2005; Torres *et al.*, 2005a), the use of smaller tissue pieces, rather than single cell suspensions (Watts *et al.*, 2000a; b), grafting cells with a syringe with a small outer diameter or a fine glass-capillary (Nikkhah *et al.*, 1994b;

Nikkhah *et al.*, 1994c; Nikkhah *et al.*, 2009) are all important parameters in optimising cell yields. Furthermore, pre-treatment of the grafting site (e.g., GDNF, FGF) might be a strategy to help the grafted cells to better survive and to direct them down an A9 phenotype (Ahn *et al.*, 2005; Torres *et al.*, 2005a; Kauhausen *et al.*, 2013). Cell survival can further be increased via pre-treatment by anti-apoptotic or antioxidant factors (e.g., caspase inhibitors, lazaroids) (Brundin *et al.*, 2000a; Brundin *et al.*, 2000b). Given the heterotopic innervation of the striatum, graft placement should be focussed on key functional targets (Mandel *et al.*, 1990). The general idea behind most of the improvements is to be gentle to the tissue, avoiding trauma, e.g. gentle mechanical dissociation, rather than producing a single cell suspension, and to transplant the cells into a non-immune-reactive environment such that the grafted cells become re-vascularized quickly.

### Circuit reconstruction

Despite evidence of partial recovery and amelioration of the lesion-induced deficits, cell replacement therapy based on fetal DAergic cells has never achieved complete recovery of all the impairments resulting from loss of striatal DA. This is also true for experiments presented in this thesis (Chapters 3.3 and 3.6) as well as those published over the last 40 years. With the exception of drug-induced rotation, none of the other tests bring behavioural performance back to the level of unoperated controls. Despite remarkable improvements by fetal nigral grafts, previous studies have failed to show full recovery on a series of behavioural tests such as operant assessment on the choice reaction time tasks (Dowd & Dunnett, 2004; Heuer *et al.*, 2013a; Heuer *et al.*, 2013c), staircase (Abrous *et al.*, 1993a; Abrous *et al.*, 1993b; Nikkhah *et al.*, 1993), sensory neglect (Dunnett *et al.*, 1987; Nikkhah *et al.*, 1993; Dowd *et al.*, 2005a), and disengage-behaviour (Nikkhah *et al.*, 1993). This is not to denigrate fetal nigral cell grafts, as they indeed show a remarkable effect on behaviour compared to lesion performance, but there is room for improvement. Especially considering that alleviation of deficits is seen with striatal grafts (transplanting developing whole ganglionic eminence (wGE) for HD models) on tests such as the staircase test, where nigral grafts do not prove to be effective (Montoya *et al.*, 1990; Montoya *et al.*, 1991).

Striatal grafts are able to alleviate many of the deficits that cannot be reinstated by nigral grafts. Although both graft-types reverse the pattern of drug-induced rotations, on other tests only striatal grafts offer recovery. Skilled paw use for example is recovered by lateral grafts of striatal tissue but not by nigral tissue when assessed in

reaching cages (Dunnett *et al.*, 1988b) or the staircase apparatus (Montoya *et al.*, 1990; Montoya *et al.*, 1991; Klein *et al.*, 2007; Rath *et al.*, 2012). Also on the lateralised choice reaction time task a similar pattern is seen with striatal grafts allowing for greater recovery than nigral grafts (Mayer *et al.*, 1992; Dobrossy & Dunnett, 1998; Brasted *et al.*, 1999b; Dowd & Dunnett, 2004; Heuer *et al.*, 2013a). Interestingly, in the striatal wGE-graft model described above, the improvement seen was not evident from the first day of testing, but rather, grafted rats improved over the consecutive weeks of post-graft testing (Mayer *et al.*, 1992; Brasted *et al.*, 1999c; 2000b). This effect has been termed '*learning to use the transplant*' (Mayer *et al.*, 1992). Extensive retraining is required for the graft-effect to emerge; in the original description grafted rats performed at control level with six weeks of testing (Mayer *et al.*, 1992; Brasted *et al.*, 2000b). Most fascinating, this training effect is very specific. In an elegant designed study, rats were trained either on the ipsilateral side to the graft first and then on the contralateral side or in the opposite pattern (Brasted *et al.*, 1999b). Training on the side opposite to the transplant led to recovery over the course of testing (re-learning) whereas lesioned rats that did not receive grafts stayed impaired. Rats that were trained on the ipsilateral side to the lesion/graft first only displayed a marginal deficit. When the sides of testing was then altered for both groups, the data showed that there was no transfer of training effect, grafted rats that were trained on the ipsilateral side first were impaired during the first days of contralateral testing, although they had received 30 days of ipsilateral training. Furthermore, also this group did then improve over the weeks of testing to a similar degree to the rats that were trained on the contralateral side first (Brasted *et al.*, 1999b). Although the training to use the transplant effect has been repeatedly reported, the level of recovery seen is dependent on the task as well. More complex behavioural tests allow for a great deal of relearning but grafted animals still do not perform at the same level as untreated controls (Brasted *et al.*, 1999b; Dunnett & White, 2006). The learning to use the transplant effect is thought to be dependent on (partial) reconstruction of the fronto-striatal circuitry (Dunnett, 1995; 1999b; 2000). Circuit reconstruction and the learning to use the transplant effect has been shown in other models as well as retina transplantation (Coffey *et al.*, 1989; 1990). Here rats received retianae transplants neonatally and were tested once the animals were mature. Although initially rats did not make use of information that was provided to the transplant, after training on a foot-shock paradigm (high motivation), rats learned to use information conveyed to the transplant (Coffey *et al.*, 1989; 1990). It can be speculated that once transplantation techniques are developed to provide the anatomical foundation of circuit reconstruction, behavioural recovery will be

improved. Furthermore, similar to the striatal transplantation model, it can be assumed that extensive post-operative training will be necessary to re-establish functional circuits and for re-learning of S-R relationship to take place.

Comparing the graft effect of the present and previous studies clearly shows the limitations of DA rich grafts that are transplanted ectopically into the denervated striatum. Here, although a great deal of recovery is achieved, grafts to not increase functional recovery with extensive testing as in experiment 3.2 rats were trained over 70 consecutive days. Although experimental evidence is still lacking, it can be speculated that (full) circuit reconstruction via grafting primary fetal VM donor tissue into the SN will allow for greater recovery if the graft makes not only the appropriate connections in the host midbrain, but also grows axons, re-innervating the lesioned striatum where appropriate connections are made. Furthermore, even if such an anatomical reconstruction of the nigro-striatal circuitry would be achieved it can be assumed that extensive post-operative training will be needed to re-establish the teaching signal conveyed by the DAergic cells for recovery to occur. In the following sections grafting parameters will be discussed further with respect to the biological foundations of greater behavioural recovery by nigral grafts.

### Behavioural assessment

Any therapeutic approach will have to undergo rigorous testing and a systematic comparison to the 'gold standard' that is currently primary fetal tissue and will have to prove beneficial in order to progress. Alternative cell sources will have to be proven to (out-)perform primary fetal tissue not only on a histological level, but also on a functional level in long term behavioural tests. However, even if 'performance' of those new cell lines is less than that of primary fetal tissue, they might still be used because, once perfected, they are advantageous in terms of less ethical challenges on tissue acquisition, standardisation of tissue production, sterility and quality control, and logistical constraints, all factors that are important for a standardised widespread application.

The type of tests used has to be carefully considered and a stable lesion is of utmost importance. It is extremely difficult to to distinguish the effect of the therapeutic intervention from unrelated factors against a moving baseline, such as can result in the presence of spontaneous behavioural recovery. In the respective chapters we have shown that bundle lesions produce not only the most severe, but importantly the most stable, impairment, both in rats and mice, and there are several tests of

drug-induced and spontaneous motor behaviour that are suitable for behavioural assessment of the bundle lesion type, both in mice (Lundblad *et al.*, 2004; Iancu *et al.*, 2005; Lundblad *et al.*, 2005; Grealish *et al.*, 2008; Francardo *et al.*, 2011; Smith & Heuer, 2011; Heuer *et al.*, 2012; Smith *et al.*, 2012a; Smith *et al.*, 2012b; Heuer *et al.*, 2013c) and in rats (Dunnett *et al.*, 1981b; Dunnett *et al.*, 1983b; a; Nikkhah *et al.*, 1994a; Dowd & Dunnett, 2004; Dowd *et al.*, 2005a; Torres & Dunnett, 2007; Torres *et al.*, 2008a).

In the second strand of this thesis (Chapters 3.4 to 3.6) we have shown that the unilateral mouse lesion model is a viable research tool when several factors are kept in mind. As discussed above, mice are not small rats, and require more attention to detail. In addition to the necessity of the testing conditions to be quiet and odour neutral, the intensive post-operative care demands a lot of attention from the researcher. Keeping this in mind, we and others have successfully utilised the mouse as a research tool and we have shown here that the unilateral lesion produces similar effects to those reported in the rat (Dowd & Dunnett, 2004; Lundblad *et al.*, 2004; Dowd & Dunnett, 2005a; Iancu *et al.*, 2005; Lundblad *et al.*, 2005; Grealish *et al.*, 2010b; Smith & Heuer, 2011; Smith *et al.*, 2012a; Smith *et al.*, 2012b). Whereas, some tests (e.g., the cylinder test) are more sensitive and useful in rats than in mice, others, such as the corridor test, seem to be more useful in the latter species (Chapter 3.4). Although knowledge about one species' behaviour is useful as a starting point in designing new behavioural tasts for a second species, systematic characterisation and validation of those tests is required before they can be confidently adopted as an assessment tool or therapeutic screen.

Behavioural testing can require large groups of animals, especially when there is large variation in the data, as is often in the case with mice. Moreover, testing can be time-consuming and labour intensive. Furthermore ethical regulations (i.e. 3Rs) make it imperative to reduce the number of animals used to the minimum numbers necessary to achieve reliable results. Initial studies, especially those using stem cells in the (near) future, should conduct their initial assessments in simple tests of motor asymmetry, including drug-induced tests of rotation for a first screening of the cells. If the cells compensate the rotational response, there is sufficient face validity to commence more complex behavioural testing. Long-term testing using stem cells derived from other species, including human cells will cause an immune response. Using immunosuppresants will make long-term operant testing almost impossible as (i) there is associated weight loss with long-term immunosuppression, (ii) cells need

to mature a long time before exerting beneficial effects, and (iii) additional food-restriction for motivation may be too tough on the animals' health. Fortunately, the recently described concept of tolerisation/desensitisation may help to conduct long-term behavioural studies, although still more data needs to be generated to address long term (>12 month) survival (Kelly *et al.*, 2009).

The lesion model described and used throughout the present thesis is most useful where the therapy is acting on the DA system, with other effects being speculative. As recently shown, GDNF overexpression was protective against 6-OHDA insult but not against cell loss caused by α-synuclein overexpression (Decressac *et al.*, 2011). Caution must therefore be taken when it comes to selection of the animal model. However, the tests used and described in this thesis have a high face and construct validity when used for cell replacement therapies based on DA. Furthermore, predictive validity is high as the model is highly responsive to DAergic drugs as l-DOPA. Whereas operant testing is highly reliable, given that the lesion is stable, simple tests of motor behaviour, that are not dependent on activation by a drug or motivation via food restriction, are often easy to implement but can be less reliable. The cylinder test is one such example where repeated testing, if not interrupted by some time, will cause the animal to cease exploring. Operant analysis of behaviour has the advantage of being fully automated and controlled by the computer, and as such is objective and less subject to experimenter bias than tests dependent upon hand testing, observation or rating measures. Furthermore, because of the large number of trials that can be gathered in a single session, standard errors of measurement can be reduced dramatically, increasing the power to detect statistically small differences in the dependent variables. Indeed, systematic multivariate designs using multifactorial analyses of variance are often only possible with this sort of data acquisition. As a consequence, operant testing has previously been shown to be more sensitive to small, but highly significant therapeutic effects, where simple tests of motor asymmetry have failed (Dowd & Dunnett, 2004).

When using animal models like the one in the present thesis it is important to keep in mind that they are models of certain aspects of the disease pathology and not models of the actual disease. No animal model does present all the clinical and neuropathological features of PD. PD does neither occur naturally in mice nor rats, most likely due to their short lifespan. Despite this, the DA depletion model used in the present work has high construct validity in the sense that DA depletion is thought to be the underlying cause of most motor deficits and most logically this model lends

itself to investigate DA replacement via the transplantation of cells. Caution must be taken when choosing the model as other disease models may be more useful depending on the question asked. Whereas GDNF has been shown to have neuroprotective effects against 6-OHDA insult to the rat brain (Rosenblad *et al.*, 1999; Kirik *et al.*, 2000a; Rosenblad *et al.*, 2000; Kirik *et al.*, 2001a; Kirik *et al.*, 2004; Dowd *et al.*, 2005b), it failed to protect DAergic neurons from α-synuclein insult and a recent clinical trial was aborted (Gill *et al.*, 2003; Lo Bianco *et al.*, 2004; Hutchinson *et al.*, 2007; Decressac *et al.*, 2011).

Furthermore, rodents differ genetically and behaviourally from humans in many aspects (primary sense used, dexterity, language, cognition). The tremor at rest that is one of the hallmark symptoms for PD has not successfully been modelled in rats or mice and the pathology seen in man is progressive spanning decades. Despite obvious advantages of the unilateral lesion model, the degeneration in man occurs bilaterally. The development of bilateral models and tests is therefore an imperative requirement to more truly assess the lesion-induced deficits and the ability of subsequent DA cell replacement to ameliorate these. Using excitotoxins or transgenic animals, studies have shown the feasibility of using cognitive and psychiatric tests as well as other sensory modalities than sight and smell, to model the human condition in the rodent (Birrell & Brown, 2000; Trueman *et al.*, 2005; Brooks *et al.*, 2006; Tait *et al.*, 2007; Tait & Brown, 2008; Dunnett *et al.*, 2012). Tests of set-shifting, olfactory discrimination, maze running, etc. will have to be developed for the bilateral rat-lesion model of PD. First studies assessing cognitive manipulations in bilateral 6-OHDA lesioned rats are emerging (Smith *et al.*, 2002), although, unfortunately, studies with partial and focal lesions do not report long-term data, therefore it cannot be assumed that the lesion produced is stable, rendering the model useless for cell replacement therapy unless such a long-term characterisation has been conducted. Here we use a model with clear advantages and limitations, and, whilst acknowledging the limitations, for the assessment of cell replacement therapies it is the best model available at the current stand of research.

In summary, behavioural testing depends on many parameters from selection of the right model to selection of the right test. For cell replacement therapy a stable lesion is imperative to disentangle the effects of the lesion from those of the therapeutic intervention. A range of tests are available covering many motor, cognitive and psychiatric domains, and it would be wise to select tests from a range of these as no single test will capture all aspects of the condition.

## Future work

There are a number of improvements that could be addressed in future experiments to further alleviate the deficit. A more widespread innervation of the striatum can be achieved via transplanting cells throughout the striatum via multiple small deposits (Nikkhah *et al.*, 1994b), larger grafts by using younger donor tissue (Torres *et al.*, 2007; Torres *et al.*, 2008a), preparation of the graft site via injection of a GDNF vector previous to transplantation (Dowd *et al.*, 2005b; Kauhausen *et al.*, 2013), and the use of additional treatments, e.g., STN lesions, DBS in the STN, or additional grafts into the SN (Baker *et al.*, 2000; Mendez *et al.*, 2000). However, despite all these promising approaches to improve graft function, the ultimate goal of circuit reconstruction is not achieved by ectopic graft placement. Homotopic graft placement would circumvent this problem and might alleviate more deficits than ectopic transplantation. Recent use of a GFP mouse has shown that fibres can reach the striatum and even the prefrontal cortex when transplanted into lesioned mice (Thompson *et al.*, 2005; Thompson *et al.*, 2009). Furthermore, using the same GFP-positive tissue, it was shown that it is the A9 component of the graft that is mainly responsible for the improvement in motor function in the unilateral rat lesion model of PD (Grealish *et al.*, 2010a). The proportion of A9 type cells within the graft was increased by the use of younger donor tissue (Bye *et al.*, 2012; Kauhausen *et al.*, 2013). With these new research tools available it will be interesting to attempt circuit reconstruction in the unilateral mouse lesion model and compare the effects of the two graft placements.

As full behavioural recovery via reconstruction of the nigro-striatal circuit has not been experimentally shown in the PD rodent model this should be the ultimate aim. In an elegantly designed disconnection experiment Dunnett and White trained rats on the classical delayed alternation task conducted in the Skinner box and showed that at least one fronto-striatal circuit is necessary for optimal task performance (Dunnett & White, 2006; White & Dunnett, 2006). In their latest study they showed functional recovery achieved with a unilateral graft in bilateral quinolinic acid lesioned rats (Dunnett & White, 2006). To display true circuit reconstruction Dunnett proposed a disconnection study which should reinstate the deficit if the recovery was due to circuit reconstruction (Dunnett & White, 2006). This is an interesting proposal that should be taken up and combined with future studies in the DA cell replacement field. A recent study by Graybiel has shown that optogenetic manipulation can be used to temporarily inhibit cells in certain (here infralimbic cortex; = rat mPFC) brain regions

(Smith *et al.*, 2012c). If cells could be transfected with the EYFP coding AAV-5 viral vector prior to grafting, and then be transplanted into the striatum/nigra, the effects of the graft could be manipulated from the outside, whereas up to date only the removal/disconnection of the graft could be done. This form of manipulation, especially once a widespread re-innervation is achieved by nigral grafts in homotopic placement, has the potential to provide compelling evidence for circuit reconstruction.

## Conclusion

Although many questions remain in terms of what cells to use and where to place the transplant, cell replacement therapies offer a viable therapeutic alternative in a subgroup of patients. Here we further characterised rodent models of PD for the assessment of what is actually impaired in the lesion and what can be repaired by our standard method of grafting. The presented data will be useful for studies to come as they provide a baseline for alternative cells/therapies to be measured against.

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# Chapter 6

# Appendix

## Solutions used

**TRIS buffered saline (stock solution)**
48g TRIS base (Sigma)
36g Sodium Chloride (Sigma)
1l distilled water
pH = 7.4

**Phosphate buffered saline**
90g di-sodium hydrogen orthophosphate (Bhd)
45 g Sodium Chloride (Sigma)
5l distilled water
pH = 7.4

**Paraform aldehyde solution (1.5%)**
90g di-sodium hydrogen phosphate (Bhd)
45g Sodium chloride (Sigma)
Paraformaldehyde (1.5%)
3300ml distilled water
pH = 7.3

**Succrose solution (25%)**
25g succrose (Fisher)
100ml phosphate buffered saline

**ABC solution**
5µl A (DAKO)
5 µl B (DAKO)
1ml 1% serum in TBS

**Quench**
10ml Methanol (Fisher)
10ml Hydrogen peroxide (VWR Prolabo BHD)
80ml distilled water

**Tris non saline (TNS)**
6g Trisma base (Sigma)
1l distilled water
pH = 7.4